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# METHODS OF STUDYING THE RESPIRATORY EXCHANGE IN SMALL AQUATIC ORGANISMS, WITH PARTICULAR REFERENCE TO THE USE OF FLAGELLATES AS AN INDICATOR FOR OXYGEN CONSUMPTION.

By H. MUNRO FOX.

*(From the Laboratory of the Marine Biological Association, Plymouth, England, and the Biological Laboratory of the School of Medicine, Cairo, Egypt.)*

(Received for publication, March 5, 1921.)

The results described below are a sequel to those already published in this *Journal* on the spontaneous aggregation of flagellates.[1] In that publication it was shown that a flagellate, *Bodo sulcatus*, forms aggregations in regions where the concentration of dissolved oxygen is an optimum for it. This optimum is less than the saturation concentration of oxygen dissolved in water under atmospheric partial pressure. The flagellates move out of regions where the oxygen concentration is above or below the optimum to gather into the optimal regions. They are positively chemotactic to a certain concentration of dissolved oxygen.

This behavior of the flagellates can be made use of to indicate changes in the concentration of dissolved oxygen due to the respiration of an aquatic organism present in the water. For if the organism under investigation be kept motionless in a suspension of the flagellates in water saturated with oxygen at the atmospheric partial pressure, the flagellates will collect into those regions where the oxygen concentration is lowered through the respiratory activity of the organism. The sizes of the aggregations of flagellates thus formed will show the relative amounts of oxygen absorbed by the different parts of the surface of the organism.

The following experiment will show how the method is applied. A small fresh water invertebrate, such as a *Chironomus* larva, is

[1] Fox, H. M., An investigation into the cause of the spontaneous aggregation of flagellates and into the reactions of flagellates to dissolved oxygen, *J. Gen. Physiol.*, 1920–21, iii, 483, 501.

placed on a slide in a few drops of liquid from a *Bodo* culture. A cover-glass, supported at its four corners by wax feet, is placed over the liquid and is pressed down so that the larva is just prevented from moving, without being injured by the pressure (Fig. 1). The flagellates in the neighborhood of the larva swim in to its surface,

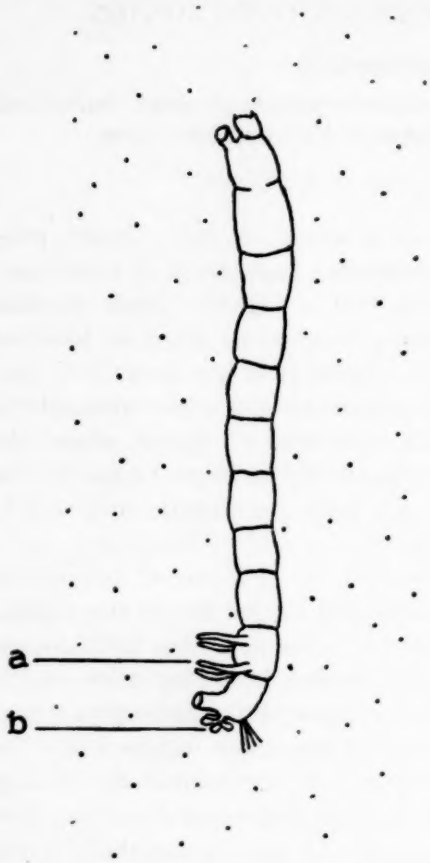


FIG. 1.

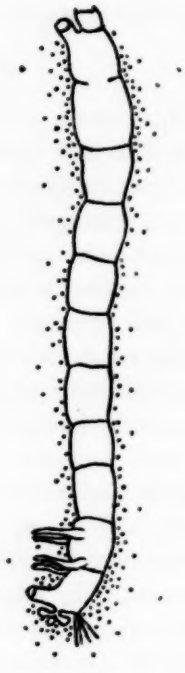

FIG. 2.

FIG. 1. *Chironomus* larva which has just been placed in a suspension of flagellates. *a*, ventral gills; *b*, anal gills. In all diagrams the density of the dots represents the density of distribution of the flagellates.

FIG. 2. The same larva shortly afterwards. The flagellates collect on the surfaces of the larva which are absorbing oxygen.

collecting there in ever increasing numbers (Fig. 2). This collection is not a surface energy phenomenon which might be caused by any solid object in the suspension, for if a larva which has just been killed by being dropped into hot water is used in place of the living larva, there is no aggregation of flagellates at its surface. The *Bodo* are simply attracted towards the surface of the living larva because in this neighborhood the respiratory activity of the insect has lowered the concentration of oxygen dissolved in the water. The reason for the collection is the same as that for the spontaneous aggregation previously described.[1] The flagellates move into a region of lower oxygen concentration caused, in the case of spontaneous aggregation, by their own respiration, in this experiment by the respiration of the larva. Here, of course, the aggregation of flagellates takes place much more rapidly than in spontaneous aggregation for the larva consumes relatively much more oxygen than the flagellates. If spontaneous aggregation occurred rapidly, it would interfere with the collection of the flagellates on the respiratory surfaces of the larva. The danger of spontaneous aggregation is, however, averted by a preliminary filtering of the *Bodo* suspension through bolting-silk. This not only removes fragments of debris from the culture jar but the exposure of the water to the air during filtration allows it to become saturated with oxygen. This greatly retards subsequent spontaneous aggregation.[1]

More flagellates collect on some parts of the insect's surface than on others. These are the most actively respiring surfaces. When the aggregation has reached certain dimensions, a clear space free from flagellates appears between it and the surface of the larva (Fig. 3). This is due to the same cause as the appearance of an area free from flagellates in the center of a spontaneous aggregation; the dissolved oxygen has been reduced to a certain low concentration at which the flagellates leave the region. The aggregation of flagellates on the surface of the larva now gradually becomes a band separated from the surface by a clear space, this happening first at those surfaces which are most actively respiring. Eventually the flagellates move out everywhere from the surface of the larva and come all to lie in a band encircling it (Fig. 4). In the case of an animal, such as a *Simulium* pupa, which respires only in one part, namely through

the walls of certain filamentous appendages, no oxygen at all being absorbed by the rest of the body surface, the aggregation and band of flagellates have the forms shown in Fig. 5. In all cases the band gradually enlarges, moving out to a stationary equilibrium position just within and parallel to the edges of the cover-glass. This is the same phenomenon as the final stage of spontaneous aggregation and band formation.

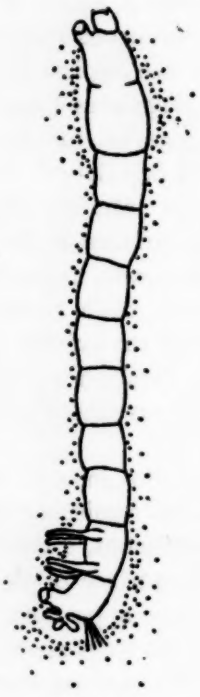

FIG. 3.

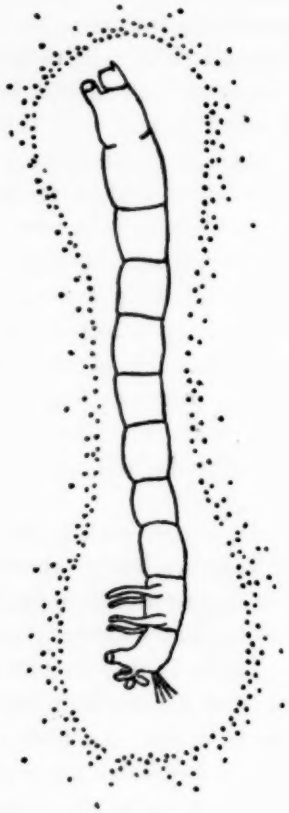

FIG. 4.

FIG. 3. The same larva a little later. The flagellates leave those surfaces of the larva which by their more active respiration have first reduced the concentration of dissolved oxygen below the optimum for the flagellates.

FIG. 4. The same larva. The flagellates have all left the surface of the larva and lie in a gradually spreading band in the zone of optimum oxygen concentration.

By this method the relative amounts of oxygen absorbed by different surfaces of a small aquatic organism can be estimated very delicately. Most oxygen is taken in at those places where most flagellates accumulate and where, later on, the aggregation first moves away from the surface in the form of a band. The use of the flagellates as an indicator in such work has the more value in that they can be cultivated either in fresh or in salt water. Throughout the present investigation the flagellate employed was *Bodo sulcatus*. It was obtained by steeping grass from the garden behind the laboratory at Plymouth in tap water.

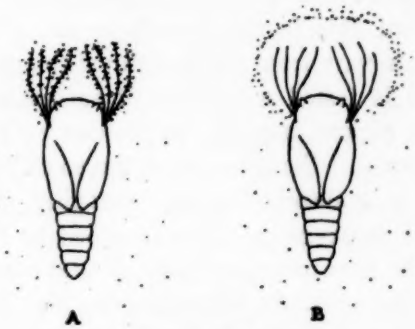


FIG. 5. *Simulium* pupa in a suspension of flagellates. A, the flagellates collect on the surfaces which are absorbing oxygen; B, later, the flagellates leave the region where the oxygen concentration has fallen below the optimum for them.

The use of flagellates for detecting the intake of oxygen by the surfaces of organisms is complementary to Engelmann's well known method of demonstrating the output of oxygen in photosynthesis by means of bacteria.[2]

The flagellates have been used so far to test the respiratory activities of several types of aquatic insect larvæ, but the work so far has been done mostly from the point of view of perfecting the methods. In the future it is proposed to examine systematically all available types of larvæ with the particular object of settling the functions of the several kinds of so-called gills. To make the meaning clearer, the work done with the red *Chironomus* larva will now be outlined.

[2] Engelmann, T. W., Neue Methode zur Untersuchung der Sauerstoffausscheidung pflanzlicher und thierischer Organismen, *Arch. ges. Physiol.*, 1881, xxv, 285.

These larvæ have on the ventral surface of the eighth abdominal segment four tube-like hollow outgrowths, in which the blood circulates. On the dorsal surface of the last abdominal segment there are four shorter hollow outgrowths, also with a blood stream through them. All these processes are thin-walled and have a thin cuticle. It is usually assumed that the chief respiratory gas-exchange takes place through the walls of these outgrowths, the so-called ventral and anal gills. When the larvæ are tested with the *Bodo* suspension, it is found that oxygen is absorbed by the whole of the general body surface of the larva except the head which has a very thick cuticle. The intensity of absorption is usually greatest in the posterior abdominal segments, but this is not invariably so. The most actively respiring segments vary from one individual to another, and when a single individual is tested a number of successive times the most actively respiring regions may vary in position each time. However, the remarkable result is the behavior of the so-called gills. No more oxygen is found to be absorbed by the "anal gills" than by the general body-surface and no oxygen at all is absorbed by the "ventral gills." The latter may be seen to project through and beyond the collection or band of flagellates without influencing it (Figs. 2 and 3).

This result can be confirmed by quite another mode of experimentation. The blood of these larvæ contains hemoglobin dissolved in it. When a larva is examined with a microspectroscope the whole body shows the oxyhemoglobin absorption bands. If a larva is now placed in water in a hollow-ground slide and covered with a cover-slip, the edges of which are sealed down with vaseline, at the end of about 20 minutes the whole body of the larva shows the reduced hemoglobin absorption spectrum. The larva does not die when this has occurred. It will remain alive with heart beating for many hours beneath the sealed cover-slip. If a small bubble of air is now allowed to enter beneath the cover-slip, so that it comes to rest up against the surface of the larva, an examination with the microspectroscope shows that oxyhemoglobin first appears inside the body of the larva at a point nearest to the bubble and thence it spreads over the rest of the body. Thus the part of the body-surface nearest to the bubble first absorbs the oxygen, and not the "gills." But a more striking result than this can be obtained. If by chance the bubble

comes to rest in contact with the "ventral gills," the first appearance of oxyhemoglobin is inside the abdominal segment nearest to the bubble and not within the "gills" themselves. This fully bears out the result obtained with the flagellates. Whatever be the function of the "ventral gills," it is not that of absorbing oxygen.

The spectroscopic method is one suited to this particular larva alone; it cannot be applied where hemoglobin is absent. The flagellate method, however, is one of general application.

At the same time that the oxygen intake was studied, the relative output of carbon dioxide by different surfaces of the body was investigated. This was done by mounting the larva on a slide in the same manner as for studying the oxygen absorption, but, in place of a suspension of flagellates, an indicator was used which changes color as carbon dioxide goes into solution, altering the hydrogen ion concentration of the water. The position and extent of the color change indicates the place and amount of the carbon dioxide output. Several indicators are available which change in color about the neutral point of water. Neutral red and rosolic acid were tried but were found unsuitable for these experiments because in the thin layer of solution between cover-slip and slide the colors are not sufficiently intense to show a sharp change. The indicator which was found suitable for use was a solution of hematoxylin with just sufficient alkali added to make it a bluish pink. In regions where carbon dioxide is being given off by the larva into the water the bluish pink color changes through orange to yellow. The color change is sharp even in the thin layer of liquid between cover-slip and slide. It can be more exactly observed when the examination is made under a very low power of the microscope, such as a 2 inch objective. All tests were discarded in which the larva defecated or in which any liquid at all came out through the anus, for the rectal fluid is alkaline.

Using the red *Chironomus* larva it was found by this method: (1) that carbon dioxide is given off by the whole body surface except by the head and the ventral gills; (2) that most carbon dioxide is given off by the posterior abdominal segments, but that the relative amounts given off by different segments vary in different individuals and in the same individual at different times; (3) that carbon dioxide is not always given off by the different surfaces of an

individual larva in the same relative amounts that oxygen is absorbed by these surfaces; (4) that the "anal gills" give off no more carbon dioxide than the general body surface; and (5) that the ventral gills give off no carbon dioxide.

It is intended to apply the flagellate method of studying the oxygen intake and the indicator method of studying the carbon dioxide output to small aquatic types of the various groups of the animal kingdom, but first of all a systematic examination will be made of aquatic insect larvæ with special reference to the functions of the so-called blood gills and tracheal gills and to the relation of closed tracheal systems to respiration.

## SUMMARY.

1. Flagellates are positively chemotactic to a certain concentration of dissolved oxygen which is lower than that in water saturated with oxygen under atmospheric partial pressure. Consequently, when a small aquatic animal is held motionless between cover-slip and slide in a suspension of flagellates in water saturated with oxygen, the flagellates are attracted to those parts of the animal which are absorbing oxygen. The relative sizes of the flagellate aggregations then show the relative activities of the different surfaces of the animal in absorbing oxygen.

2. Applying this method to the red *Chironomus* larva it was found that the animal respires by the whole body surface except by the head and the "ventral gills" and that the relative intensity of oxygen intake by the different parts of the body varies in different individuals and in the same individual at different times.

3. The absence of oxygen intake by the "ventral gills" was confirmed with the microspectroscope. In oxygen-free water all the hemoglobin of the blood becomes reduced. When an air bubble is now introduced so that it touches the "ventral gills" oxyhemoglobin first appears in the nearest body segment to the bubble, not in the "gills."

4. When a small aquatic animal is held motionless between cover-slip and slide in a solution of an indicator which changes color about the neutral point of water the relative extent of color change at different surfaces of the animal's body indicates the relative amounts of carbon dioxide given off by these surfaces.

5. Using this method with the red *Chironomus* larva similar conclusions were reached for carbon dioxide output as for oxygen intake.

The work was done in August and September, 1919, at the Laboratory of the Marine Biological Association, Plymouth. I wish to thank the Director and staff for their constant kindness and assistance. I wish also to thank the trustees of the Ray Lankester Fund for having nominated me to an Investigatorship and Sir Ray Lankester, K.C.B., F.R.S. for having lent me a microspectroscope.

# THE PHAGOCYTOSIS OF SOLID PARTICLES.

## III. Carbon and Quartz.

By WALLACE O. FENN.

*(From the Laboratory of Applied Physiology, Harvard Medical School, Boston.)*

(Received for publication, January 15, 1921.)

It has been shown by Haldane (1) and by Mavrogordato (2) that silicious dust when inhaled tends to remain in the lungs, causing phthisis. Coal dust, on the other hand, tends to move out of the lungs and is, therefore, harmless. The different behavior of silicious and carbonaceous dusts in the lungs is also the cause of the abnormally high mortality from tuberculosis among silicious miners and the abnormally low mortality among coal miners (3). Without going into a discussion of the mechanism of dust removal from the lungs, it is sufficient to state that the first step appears to be always the ingestion of the dust particles by phagocytic cells in the alveoli. It seemed probable, therefore, that a study of the phagocytosis of carbonaceous and silicious particles would show that the former are ingested more readily than the latter, in agreement with the clinical facts, and might throw some light on the cause of this difference. This was found to be true.

It became at once evident, in undertaking a comparison between the rates of phagocytosis of carbon and quartz particles, that the usual method of incubating them together with the leucocytes in a common suspension might yield nothing more than a comparison of the relative chances of collision of the two kinds of particles with the leucocytes. It has, in fact, been shown in two preceding papers on the phagocytosis of quartz (4) and carbon (5) particles that the more rapid ingestion of large particles by leucocytes compared to small ones can be accounted for quantitatively by the fact that a large particle moves faster when stirred up in a suspension with leucocytes, and therefore comes into collision with more cells in a given time. In this paper the same methods will be applied to a

study of the comparative rates of phagocytosis of carbon and quartz particles to determine whether or not carbon is ingested by leucocytes more readily than would be predicted from the calculated chances of collision.

The method of calculating $R$, the chance of collision, and the experimental procedure are described in the earlier papers (4, 5). It must

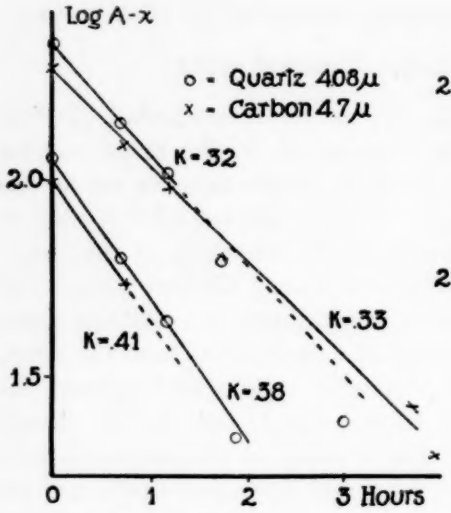


FIG. 1.

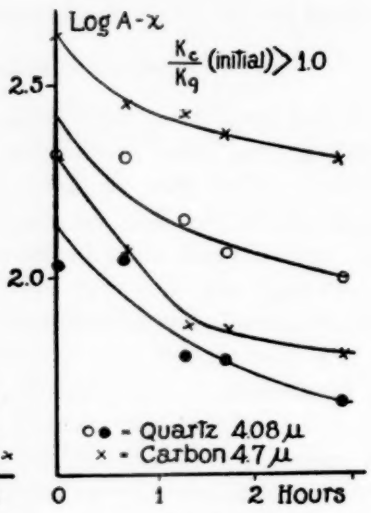


FIG. 2.

FIG. 1. Ordinates represent logarithms of the number of particles outside the leucocytes in 0.02 c. mm. of the suspension. Curves show that the actual rates of ingestion of the quartz and carbon were nearly equal. Quartz, being, heavier, collides more often with the cells. The corrected ratio, $\frac{K \text{ carbon}}{K \text{ quartz}} = \frac{1.06}{0.36} = 3$, shows that carbon is taken up 3 times as readily as quartz. Two concentrations of both quartz and carbon were used, the higher concentration being twice the lower. See Table II.

FIG. 2. Ordinates and abscissae as in Fig. 1. Curves showing the rates of ingestion of quartz and carbon particles. If the experimental points for quartz are accurate the initial $K = 0$. Assuming that the first two points on the quartz curves are erroneous, as seems most probable, the initial ratio, $\frac{K \text{ carbon}}{K \text{ quartz}}$, is seen to be at least $> 1$. Even interpreting the curves thus in favor of the quartz, the corrected ratio is 2.8, showing that the carbon is taken up at least 2.8 times as readily. Two concentrations (in the proportion of 1 to 2) of both quartz and carbon were used.

suffice here to state that $R$ is proportional to the difference between the velocities of leucocytes and particles, $Vp - Vc$, and to the square of the sum of the diameters of cells and particles, $(C + P)^2$. The latter factor allows for the fact that a larger particle makes a larger target. The rate of phagocytosis is measured by $K$ of a monomolecular reaction and is equal to the slope of the straight line obtained by plotting against time the logarithms of the *number of particles not yet ingested*, as determined by frequent counts on an ordinary hemocytometer. The cell suspensions were obtained from peritoneal exudates in rats. The phagocytic mixtures were rotated slowly on a revolving drum during incubation to prevent settling out of the cells or particles.

TABLE I.

*Calculated Chances of Collision, R.*

| Nature of Particle. | Diameter. | R. |
|---|---|---|
| | μ | |
| Carbon | 3.2 | 144 |
| | 4.7 | 248 |
| Quartz | 2.4 | 130 |
| | 4.08 | 697 |
| | 4.63 | 1299 |

The results of one such experiment are plotted in Fig. 1. Since ordinates represent the logarithms of the number of particles counted at time, $t$ (abscissæ), *outside* the leucocytes, the steeper the slope of the curve the more rapid the phagocytosis. A straight line in this figure, *i.e.*, a constant $K$, indicates that the same percentage of the number of collisions occurring between cells and particles is resulting in ingestion throughout the experiment. As has previously been pointed out, it is only the *initial slope*, in cases where $K$ is *not* constant, which is expected to agree with the theoretical predictions. In Fig. 1, the initial $K$'s of the two carbon experiments are 0.33 and 0.41, and of the quartz, 0.38 and 0.32 the averages being 0.37 and 0.35 respectively; *i.e.*, carbon is taken up $\frac{0.37}{0.35}$ or 1.06 times as fast as the quartz. Reference to Table I, however, in which are tabulated the chances of collision of the three quartz and two carbon suspensions used in

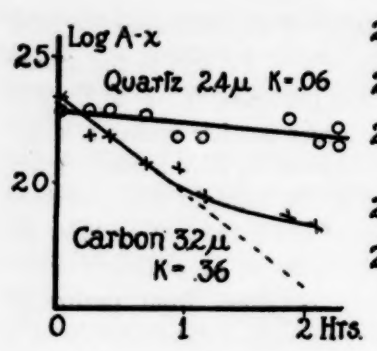


FIG. 3.

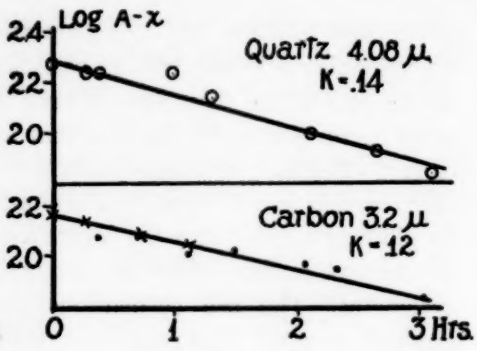


FIG. 4.

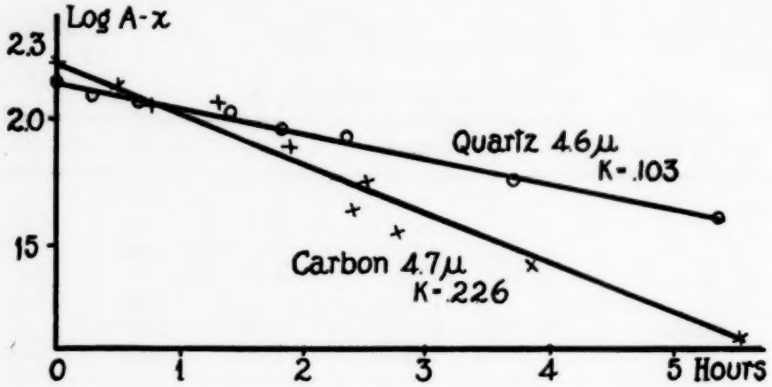


FIG. 5.

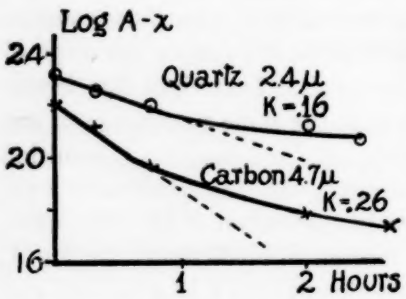


FIG. 6.

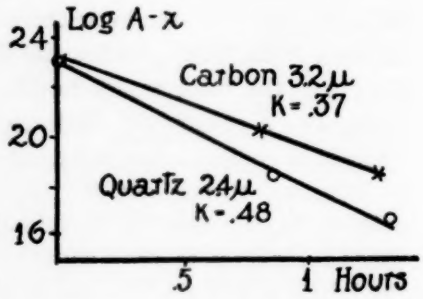


FIG. 7.

FIG. 3–7. Curves showing comparisons of rates of ingestion of quartz and carbon particles of various sizes by the suspension method. The logarithms of the number of particles in 0.02 c. mm. not yet ingested are plotted as ordinates against time in hours as abscissae. See Table II for the results of the comparisons when corrected for the chances of collision.

these experiments, shows that these carbon particles (4.7$\mu$) should collide with the cells only $\frac{248}{697}$ or 0.36 times as often as the quartz (4.08$\mu$). It may be concluded, therefore, that carbon is ingested $\frac{1.06}{0.36}$ or 3 times as readily as quartz.

Results of all the other available comparisons between quartz and carbon are plotted in Figs. 2 to 8 and tabulated in Table II. In each experiment the initial $K$ only was used in comparison unless $K$ was constant throughout. The last column in Table II shows the number of collisions necessary for ingestion of one quartz particle if every collision with a carbon particle results in ingestion; *i.e.*, the

TABLE II.

*Comparison of Rates of Phagocytosis of Carbon and Quartz by "Suspension" Method.*

| Fig. | Diameter of particles. | | Initial $K$. | | Ratio* $\frac{K \text{ carbon}}{K \text{ quartz}}$. | | |
|---|---|---|---|---|---|---|---|
| | Carbon. | Quartz. | Carbon. | Quartz. | Observed. | Calculated. | Corrected. |
| 1 | 4.7 | 4.08 | 0.37 | 0.35 | 1.07 | 0.36 | 3.0 |
| 2 | 4.7 | 4.08 | 0.03 | 0.03− | 1.0+ | 0.36 | 2.8+ |
| 3 | 3.2 | 2.4 | 0.36 | 0.06 | 6.0 | 1.1 | 5.4 |
| 4 | 3.2 | 4.08 | 0.12 | 0.14 | 0.87 | 0.21 | 4.1 |
| 5 | 4.7 | 4.6 | 0.226 | 0.103 | 2.2 | 0.19 | 11.5 |
| 6 | 4.7 | 2.4 | 0.26 | 0.16 | 1.6 | 1.9 | 0.8 |
| 7 | 3.2 | 2.4 | 0.37 | 0.48 | 0.77 | 1.1 | 0.7 |

Average = 4.0

* The calculated ratios are the ratios of the chances of collision for the sizes of particles used as given in Table I. The corrected ratio is the quotient obtained by dividing the observed ratio by the calculated ratio.

value of the ratio $\frac{K \text{ carbon}}{K \text{ quartz}}$. In five out of seven experiments carbon is taken up 2.8 to 11.5 times as readily as quartz. In two instances only, due to some uncontrolled factor, quartz is taken up slightly more readily than carbon. The average of all shows that carbon is taken up 4 times as readily as quartz.

It should be observed that it is assumed that a large particle is taken up as easily as a small particle if the number of collisions are equal. That this is true, at least within limits of error of these experiments, was shown in the two previous papers (4, 5). It would appear

from these figures that there is great variation in different experiments in the relative rates of ingestion of carbon and quartz, the ratio varying from 0.7 to 11.5. That this ratio may vary with the condition of the cells, even in the same experiment, will be shown later by another method.

Another contributing explanation is perhaps the agglutination of the carbon. In some experiments the carbon has been observed to be more stable than in others. This factor could always be controlled, however, by observation of the samples which were removed from the incubation mixtures at intervals for counting. Whenever any considerable agglutination was observed the experiment was discarded. With quartz, however, there was seldom any agglutination, and this is the most characteristic difference between the behavior of the two kinds of particles. Thus quartz can be thrown down repeatedly by centrifugalization, and when resuspended in water the particles are perfectly discrete. This cannot be done with carbon unless it is stabilized with acacia. This suggests that the cause of the more rapid agglutination may also be the cause of the greater speed of phagocytosis. One might suppose that if carbon agglutinates with carbon readily, it will also agglutinate readily with cells, whereupon ingestion promptly occurs.

*Comparison of Quartz and Carbon by the "Film" Method.*

Before proceeding to a discussion of this hypothesis, however, some experiments will be reported in which the rates of ingestion of carbon and quartz were compared by another method, which will be referred to as the "film" method. By this method, unlike the "suspension" method described previously, phagocytosis was allowed to proceed in a thin film between the slide and cover slip. Both cells and particles immediately settled out. The leucocytes crept about on the slide and ingested the particles.

A cover-slip was supported at its center and four corners by fragments of another fairly thick cover-slip. While held down with a small weight it was sealed at the corners with collodion. The weight prevented the cover-slip from floating up on the collodion, and made the distance from slide to cover uniform throughout. A thick suspension of cells with equal numbers of carbon and quartz particles

was then allowed to run under the cover-slip and the edges were sealed with paraffin to prevent evaporation. The slide was at once placed on a warm stage at 37°C. and frequent counts were made of the number of free particles of quartz and carbon over a given area. The areas were measured by a disc micrometer in an ocular ruled in squares. It was customary to count sixteen such areas for each point.

The advantages of this method are:

1. The chance of collision depends merely upon the size which the particle offers as a target for the cells; i.e., $(C + P)$ which is so small a factor that it is almost negligible.[1]

2. Agglutination of particles is impossible.

3. Both quartz and carbon particles can be incubated together in the same film so that the number of active cells is identical at any one time.

The only disadvantage of the method is that the cells are injured by contact with the glass slide, so that the time curves do not follow the law for a monomolecular reaction as they otherwise would. Thus the cells are observed during the course of the reaction to spread out on the glass, becoming vacuolated and transparent and ceasing their activities.

Phagocytosis with the film method presents an interesting picture, as shown in the photographs (Figs. 8 and 9). Nearly all the cells are in active motion. The activities of one cell were recorded for 3 minutes during which time it refused one quartz particle and one carbon particle, and ingested three carbon particles, nearly reaching a fourth. No evidence was obtained that cells can sense particles from a distance, as reported by Commandon (6) for leucocytes ingesting starch grains, and by Schaeffer (7) for amebæ in proximity to carbon and glass. All the meetings seem to occur purely by chance. Sudden movements of leucocytes for a distance 2 or 3 times their own length have frequently attracted attention, as if the cell had been under

[1] $C$ = diameter of cell, $P$ = diameter of particle. This is merely the target factor from the formula for the chances of collision as used in the suspension method. In this instance, however, the chance of collision is $(C + P)$, not $(C + P)^2$, because motion is confined to two dimensions.

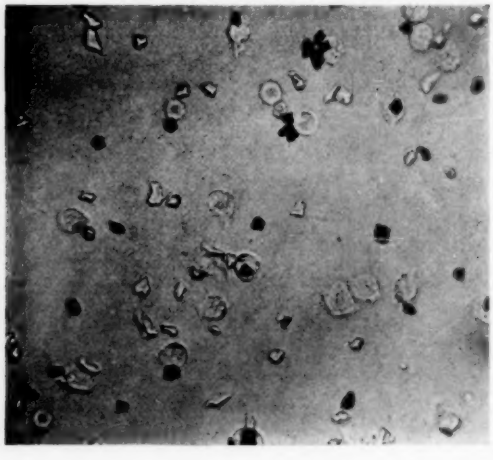

FIG. 8.

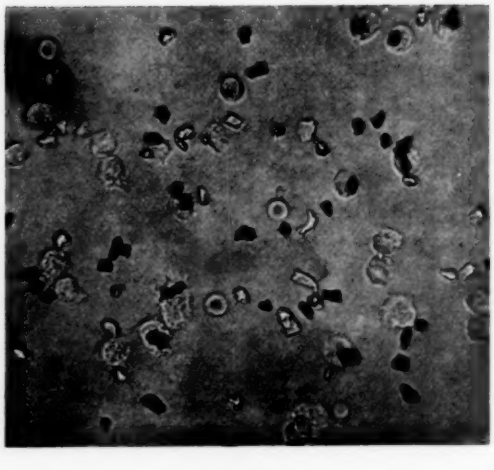

FIG. 9.

FIGS. 8 and 9. Photographs of quantitative comparison of the rates of phagocytosis of quartz and carbon particles by leucocytes of rats. The leucocytes are actively ameboid and many contain carbon particles. None of the available quartz particles, however, has yet been ingested. Attention is directed to the uniformity in the sizes of the quartz and carbon particles.

tension by some long contracting pseudopod[2] which had suddenly succeeded in pulling the cell loose from its contact with the slide. Toward the end of an experiment the cells are usually aggregated in groups, mostly heavily laden with particles. This clumping is also found, apparently to the same extent, in a control preparation from which particles are omitted. Often cells are so full of particles that close inspection is necessary to see protoplasm at all.

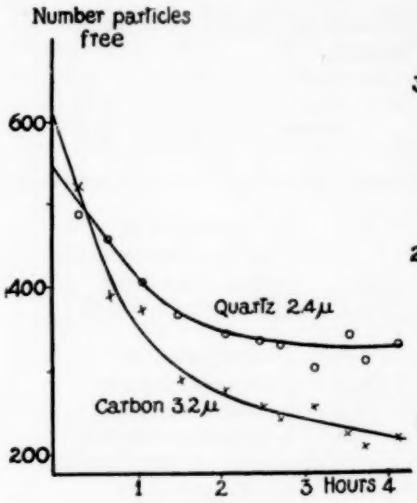


FIG. 10.

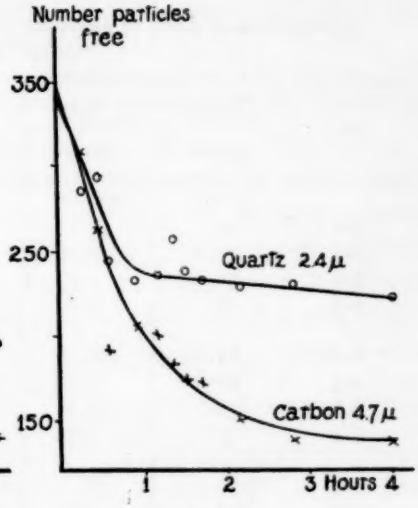


FIG. 11.

FIGS. 10 and 11. Comparison of phagocytosis of carbon and quartz by the film method. The numbers of particles not yet taken up in a given area are plotted as ordinates against time in hours as abscissae. Here no appreciable allowance need be made for the chances of collision, and carbon is obviously ingested more rapidly than quartz. See Table III for further analysis.

The results of two typical experiments of this sort are plotted in Figs. 10 and 11. Ordinates represent the number of particles of quartz or carbon counted over a given area which are still outside the cells. Time is plotted as abscissae. Inspection shows that the carbon is taken up more readily than the quartz. The question is how much more readily. Here the data can be analyzed with more

[2] Such pseudopods have been described by Kite (Kite, G. L., *J. Infect. Dis.*, 1914, xv, 319).

assurance than with the suspension method. From Figs. 10 and 11 the number of particles ingested during each hour of the experiment has been determined graphically. This value divided by the average number of particles present outside the cells during that hour gives the percentage of particles taken up during the hour. These figures are recorded in Table III.

TABLE III.

*Comparison of Rates of Ingestion of Quartz and Carbon Particles by "Film" Method.*

| Hrs. | Particles ingested per hr. | | Experimental ratio* = $\frac{\text{per cent } C}{\text{per cent } Q}$. | |
|---|---|---|---|---|
| | Carbon. | Quartz. | | |
| | *per cent* | *per cent* | | |
| 0–1 | 54 | 29 | 1.9 | Experiment 8. |
| 1–2 | 22 | 19 | 1.1 | Carbon 3.2 microns |
| 2–3 | 15 | 4.5 | 3.3 | Quartz 2.4 microns |
| 3–4 | 13 | 1.6 | 8.1 | Theoretical ratio 1.07 |
| 0–1 | 53 | 35 | 1.5 | Experiment 9. |
| 1–2 | 27 | 4.3 | 6.3 | Carbon 4.7 microns |
| 2–3 | 12 | 2.6 | 4.6 | Quartz 2.4 microns |
| 3–4 | 2.2 | 2.2 | 1.0 | Theoretical ratio 1.20 |
| 0–1 | 57 | 27 | 2.2 | Experiment 10. |
| 1–2 | 27 | 5.9 | 4.6 | Carbon 4.7 microns |
| 2–3 | 3.5 | 1.2 | 2.9 | Quartz 4.6 microns |
| | | | | Theoretical ratio 1.01 |
| 0–1 | 44 | 15 | 2.9 | Experiment 11. |
| 1–2 | 22 | 11 | 2.0 | Carbon 2.7 microns |
| 2–3 | 10.5 | 4.9 | 2.1 | Quartz 2.6 microns |
| | | | | Theoretical ratio 1.01 |

* The experimental ratios are the ratios of the numbers of particles of carbon and quartz ingested per hour expressed in per cent of the average number present during that hour and $= \frac{\text{per cent Carbon}}{\text{per cent Quartz}}$. Data were obtained graphically from Figs. 10 and 11 for Experiments 8 and 9 and from similar unpublished figures for Experiments 10 and 11. The theoretical ratios are $\frac{R \text{ carbon}}{R \text{ quartz}}$. $R = C + P$ or the chance of collision, where $C = 9\mu$, the diameter of the leucocytes, and $P =$ the diameter of the particle.

The ratio of these values $\left(\frac{\text{per cent Carbon}}{\text{per cent Quartz}}\right)$ for carbon and quartz (last column) is then a measure of the greater speed of ingestion of carbon. Strictly these figures should now be corrected by dividing by the ratio $\frac{(C+P)\text{ carbon}^1}{(C+P)\text{ quartz}}$ which is a measure of the relative chances of collision. Since the value of this factor is small (1.07 and 1.2), this correction has not been applied, but the necessary factor for correction is given for each comparison.

It is a very significant fact that this $\frac{\text{per cent Carbon}}{\text{per cent Quartz}}$ ratio increases after the 1st hour. This means that in comparison to carbon it is relatively harder to ingest quartz toward the end of the experiment than at the beginning. Thus, in Experiment 8 (Fig. 10), if every meeting between a cell and a carbon particle results in ingestion throughout the experiment, we may conclude that at the beginning of the experiment two meetings with quartz are necessary for ingestion, and at the end of the experiment, eight meetings. Eventually, carbon also is refused and the ratio $\frac{\text{per cent Carbon}}{\text{per cent Quartz}}$ necessarily decreases again. This happens in Experiment 9 (Fig. 11). If the phagocytic activity of the cells remained the same throughout an experiment, the percentage of carbon particles ingested during each hour would be constant. The rate of decrease of this percentage may, therefore, be taken as a measure of the rate at which the activity of the cells decreases. Thus, in Experiment 8, the phagocytic activity of the cells after 3 hours is only $\frac{13}{54}$ or 24 per cent of the original. This may be due to contact with the glass, agglutination of cells, decreased capacity of cells, or other factors.

Data from Experiments 10 and 11 are also included in Table IV. The former is the only exception to the rule that the ratio $\frac{\text{per cent Carbon}}{\text{per cent Quartz}}$ increases after the 1st hour. In this experiment both quartz and carbon (particularly quartz) were taken up more slowly even during the 1st hour than in the other three experiments, and it therefore seems probable that the decrease in the ratio in

Experiment 11 may correspond to the decrease *following* the increase in Experiments 9 and 10.

*The comparative rates of ingestion of carbon and quartz particles depend, therefore, upon the condition of the cells.* Sometimes preparations of carbon and quartz have been made in which there was practically no ingestion of quartz. This may be seen in the photographs, Figs. 8 and 9, where no quartz is seen inside the cells though there is plenty available.

As far as the writer is aware the experiments in this paper constitute the first quantitative comparison of the effects of different kinds of solid substances on living cells. Even qualitative comparisons are limited. Aside from the effects of carbonaceous and silicious dusts in the lungs the only recorded observations seem to be the mere statement of Commandon (6) that starch is ingested by leucocytes more readily than carbon. Schaeffer (7) endeavored to compare the ingestion of carbon and glass particles by amebae but neither was ingested.

### *Phagocytosis of Carbon and Quartz by Sponge Cells*

This experiment was done at the Marine Biological Laboratory, Woods Hole, with a small marine sponge, *Grantia*. The sponge was squeezed into a test-tube, and a thick suspension in sea water of active cells was obtained. 8 parts of this suspension were mixed with 4 parts of sea water concentrated to twice its normal strength by boiling, 1 part of 0.2 M borate mixture (pH 7.5), 1 part 10 per cent acacia neutralized with sodium hydroxide, and 2 parts of a suspension of 3.2 micron carbon particles and 2.4 micron quartz particles in distilled water. The result is a suspension of cells, quartz, and carbon in normal sea water plus 0.6 per cent acacia to stabilize the carbon and quartz, the alkalinity being approximately the same as that of normal sea water.

A sample of this mixture was allowed to run under a cover-slip supported as for the film method and sealed with paraffin. The cells were slowly ameboid and ingested the particles rather sluggishly compared to leucocytes. After 5 hours at room temperature, counts were made of the number of quartz and carbon particles found inside

the cells. Only solitary cells were included in the count as it would be easy to overlook the colorless quartz particles in the aggregated cells. All the solitary cells in each field were examined. In all, 9 quartz particles and 59 carbon particles were found ingested by some 200 to 300 cells which were examined.

Since there were approximately twice as many carbon particles as quartz present, it may be concluded that carbon is ingested by sponge cells $\frac{59}{9 \times 2}$ or 3 times as readily as quartz. This agrees well with the results with leucocytes. The sponge cells were not considered sufficiently phagocytic to justify more quantitative experiments.

## DISCUSSION

Whatever hypothesis is adopted to explain the more rapid ingestion of carbon, it must be concluded that there is for some reason greater attraction between the cell substance and the carbon than between the cell substance and the quartz. In view of the high adsorptive capacity of carbon this is not surprising, nor is this difference between different kinds of solid particles without its parallel in inorganic systems. Similar examples of selective "wetting" of solid particles are found in the flotation processes for the separation of different kinds of ores (8), which depend upon the fact that, in general, particles of the heavy metals have a greater affinity for oil and air phases than for water, and are thus floated to the surface while the worthless gangue particles such as silicates remain in the water and sink.

Rhumbler (9) remarks that the rhizopod *Euglypha*, in forming its shell of small particles, is able to distinguish between different materials. In imitating this he rubbed up coal and quartz particles in various oils and sprayed them into 70 per cent alcohol. In such instances the coal remains on the inside of the oil drops and the quartz collects in the surface. This observation being of considerable interest for these experiments has been repeated quantitatively with oil drops in 70 per cent alcohol and chloroform drops in water. In all cases the carbon collects in the interface between the phases more rapidly than the quartz.

The results with chloroform are plotted in Fig. 12. Similar results are obtained with cottonseed oil drops in 70 per cent alcohol or water. Moreover, if a mixture of equal numbers of quartz and carbon particles in water is warmed up with phenol above the critical solution tempera-

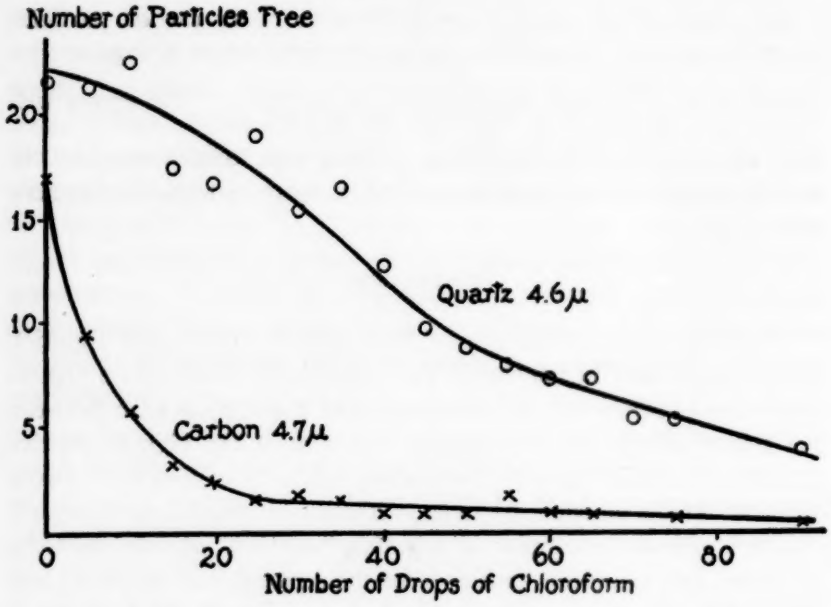


FIG. 12. Analogy to selective phagocytosis of carbon in a chloroform-water system. To 2 cc. of a suspension of carbon particles 4.7$\mu$ in diameter and quartz particles 4.6$\mu$ in diameter, chloroform is added 5 drops at a time. After each addition the mixture is shaken vigorously, allowed to stand a few minutes until the chloroform drops have settled to the bottom, and counts made of the number of carbon and quartz particles left. These figures are plotted as ordinates against the number of chloroform drops added as abscissae. Both particles accumulate in the chloroform-water surfaces, the carbon, however, much more rapidly than the quartz.

ture, on subsequently cooling the mixture, the majority of the carbon is found in the phenol phase and the majority of the quartz in the water phase, as shown by actual counts of the number of particles of each in the two phases, as follows:

| | In phenol phase. | In water phase. |
|---|---|---|
| Carbon | 44 | 5 |
| | 60 | 20 |
| Quartz | 5 | 40 |
| | 2 | 9 |

Extensive qualitative experiments of a similar nature have been carried out by Reinders (10) and by Hofmann (11). Both agree that particles of carbon are among those most easily adhering to droplets of oil, chloroform ether, etc., but neither used quartz particles for comparison.

The theoretical distribution of solid particles between two liquid phases according to the surface tension relations has been excellently summarized by Thompson (12). In a general way it may be said that in a carbon-oil-water system the lower the potential energy of a carbon-oil surface, the greater the chance of the carbon being in the oil, and *vice versa*. By collecting in the interface the oil-water surface is reduced. Therefore, the higher the oil-water tension, other quantities being equal, the greater the chance of the carbon being in the boundary. By collecting in the interface the carbon decreases the potential energy of the oil-water surface. This is presumably the reason for the emulsifying power of lampblack on water-kerosene systems reported by Moore (13).

Although the more rapid ingestion of carbon by leucocytes could not be predicted from the selective wetting of carbon by oil, phenol, and chloroform drops[3] the same principles of surface tension which explain the inorganic phenomenon can be applied to an explanation of the biological fact. It may, therefore, be argued that carbon would be taken up by leucocytes more rapidly than quartz, if the potential energy of a carbon-serum surface were greater than the potential

[3] The fallacy of predicting a surface tension of a particle in one medium from its surface tension in another is shown convincingly by the behavior of particles of manganese dioxide and manganese silicate. The former are taken up less rapidly than the latter by drops of chloroform and paraffin oil, but about twice as rapidly by sponge cells and in some experiments at least 20 times as rapidly by rat leucocytes.

energy of a quartz-serum surface, *other things being equal.* It is interesting that this is exactly the condition which should also cause more rapid clumping of the carbon than the quartz, since clumping involves a decrease of surface. It has already been mentioned that this difference in stability is the one outstanding distinction between quartz and carbon.[4]

According to Bredig (14) a low surface tension between a particle and a liquid should correspond to a high electric charge, thus reconciling electrical and surface tension theories of coagulation. If this theory is correct quartz should carry a higher charge than carbon, and the available evidence in the literature indicates that this is so. Thus, Whitney and Blake (15) measured the charge on quartz particles by kataphoresis and found a rather high negative charge of 0.042 volts. Rona and Michaelis (16) found that H and OH ions were equally adsorbed by carbon, and concluded, in consequence, that carbon must carry no electric charge. This opinion was, moreover, corroborated by a statement of Freundlich's (which they quote in a footnote) to the effect that this lack of a charge on carbon accounts for difficulty which he had experienced in trying to measure the kataphoresis of carbon. Rona and Gyorgy (17) were led to the same postulate concerning carbon by measurements of the effect of non-electrolytes on the speed of settling.

Some attempts were made to confirm this difference between the electric charges on quartz and carbon qualitatively by measuring the stability of the suspensions in various concentrations of sodium hydroxide and hydrochloric acid. Uniform suspensions, 2 to 3 microns in diameter, of quartz and carbon were used, and the degree of coagulation in the different solutions was determined by counts of the total number of particles, aggregate and single, in the case of carbon, and of the number of clumps in the case of quartz. The coagulation of quartz was so slight even in the most effective concentration of hydrochloric acid that there was scarcely a measurable decrease in the total number of particles. Both suspensions showed greatest stability in neutral and alkaline solutions and both showed an

[4] In agreement with this hypothesis is the fact that manganese dioxide is ingested by leucocytes with extraordinary rapidity compared to manganese silicate, and is also much less stable in suspension.

optimum coagulation between 0.0003 and 0.005 M hydrochloric acid (probably at 0.001 in the case of quartz). The results, therefore, neither prove nor disprove the hypothesis although they do indicate that carbon carries a negative charge contrary to the evidence from the literature. Also, the greater stability of quartz as compared to carbon in all concentrations of hydrochloric acid appears to prove that stability (and hence probably phagocytosis) is conditioned by some factor besides the electric charge, presumably the surface tension.

There are many valid objections (McClendon (18), Hyman (19), and Loeb (20)) to the explanation of ameboid movement by surface tension changes on account of the more or less rigid ectoplasm of the cell. In so far as phagocytosis is limited by ameboid movement, these objections also apply to the explanation of ingestion of particles by surface tension. The ingestion of quartz in these experiments, however, is not limited by the ameboid movement of the leucocytes which is sufficiently vigorous to cause ingestion of carbon at a more rapid rate. It is, therefore, a question of molecular attractions between particle and cell, and these are best expressed in terms of surface tension.

The analogy between this hypothesis and the sometimes parallel effects of opsonins and agglutinins has been suggested to the writer by Dr. W. B. Cannon. Thus, Bull (21) in a series of papers has brought out the fact that bacteria injected into the circulation are removed more rapidly, apparently by phagocytosis, when agglutinated by immune serum. Zinsser (22) quotes Ottenberg as authority for the statement that phagocytosis of foreign red cells in the circulation after blood transfusion occurs only when the patient's serum has an agglutinative action on the donor's cells. Likewise, Ledingham (23) has pointed out "that the opsonin-bacteria like the agglutinin-bacteria are probably in a more precipitable condition than non-sensitized bacteria. They are, in fact, as I have frequently observed, in a condition of extremely fine aggregation, and if the sensitizing fluid has marked agglutinating powers the sensitized organisms are frankly clumped." From this evidence it appears that bacteria as well as solid particles are more readily ingested when easily agglutinated.

SUMMARY

1. The rates of ingestion of quartz and carbon particles by leucocytes, when both are in suspension in serum, was compared with the availability of the two particles as predicted from the calculated chances of collision with the leucocytes, and it was shown that carbon is ingested about 4 times as readily as quartz.

2. The greater ease of ingestion of carbon was verified by a new method of measuring phagocytosis, described as the film method in which the cells ingest particles as they creep about on a slide.

3. The relative rates of ingestion of carbon and quartz depend upon the condition of the cells, the difference increasing as the phagocytic activity of the cells decreases.

4. Sponge cells also ingest carbon about 3 times as readily as quartz.

5. The hypothesis is suggested that the cause of the more rapid ingestion of carbon may be identical with the cause of the greater instability of the carbon suspensions.

6. An inorganic analogy to this selective phagocytic action is offered.

7. The application to opsonins and agglutinins is discussed.

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# STUDIES ON ENZYME ACTION.

## XIX. The Sucrolytic Actions of Bananas.

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### INTRODUCTION.

The change of starch into simpler carbohydrates such as glucose, sucrose, etc., or the reverse reaction, is a general phenomenon which accompanies the growth and development (so called ripening) of many edible fruits and also of a number of vegetables. For example, the starch of apples and of bananas in the maturing of the fruit becomes converted into simpler carbohydrates, while the sugars of maize kernels and of peas are converted into starches.[1] In the belief that enzymes and enzyme actions play an important, if not a predominating, rôle in these chemical changes, a study was begun of the enzymes which might be expected to be involved in these reactions. The banana (*Musa sapientum*) was chosen as a typical example of a fruit in which such changes occur because of the possibility of readily obtaining large quantities of the unripe fruit, the rapidity with which it can be made to ripen, the fact that it ripens when separated from the growing plant, and the extensive change from starch into glucose, invert sugar, and sucrose which accompanies the ripening.

### *Previous Work.*

For the purpose in view, the chemical composition of the unripe and ripe bananas is of interest. The results given by Gore[2] are perhaps the most satisfactory and may be quoted as follows: As a result of ripening a number of bananas, a loss in weight of 3.88 per cent was

[1] *Cf.* Sherman, H. C., Chemistry of food and nutrition, New York, 1918, 12.

[2] Gore, H. C., *J. Agric. Research*, 1914–15, iii, 187.

observed. When unripe, the fruit consisted of 41.72 per cent peel and 58.28 per cent pulp; when ripe, 37.85 per cent peel and 62.15 per cent pulp. During ripening, the main changes in the peel consisted of a decrease of 5 per cent in water content and the transformation of two-thirds of the starch into sugar. In the pulp, the starch content changed from 13.15 to 2.40 per cent, the reducing sugars from 0.37 to 10.34 per cent, and the sucrose from 0.38 to 1.52 per cent, while the water content increased 1.6 per cent.

Enzyme studies on bananas have been carried out from time to time. The most complete investigations were published by Tallarico,[3] who reported the presence of sucrase, amylase, protease, and catalase, the absence of lipase, while the results for tyrosinase were not conclusive, and by Bailey,[4] who found amylase, sucrase, raffinase, protease, lipase, and peroxidase, but not maltase, dextrinase, or lactase. Sucrase and amylase are mainly of interest in connection with the present work. Both Tallarico and Bailey proved qualitatively the presence of sucrase in bananas, considerably more in ripe than in unripe ones. With regard to the amylase, the results were not so satisfactory. The errors in the method used by Tallarico apparently were as great as the observed actions. Bailey's results were obtained with the iodine test under certain conditions.

### *Methods of Testing.*

The amount of amylase or of sucrase action was determined in most of the experiments by the estimation of the reducing sugars formed.[5,6] In carrying out the estimations, from 2 to 10 cc. portions of the mixtures tested were used, depending upon the amount of reducing substance present.

In a few experiments the starch-splitting actions were followed by adding iodine dissolved in potassium iodide solution to the mixtures.

Sodium hydroxide and hydrochloric acid solutions were employed to bring the mixtures to definite hydrogen ion concentrations. In

[3] Tallarico, G., *Arch. farm. sper. e sc. aff.*, 1908, vii, 27, 49.

[4] Bailey, E. M., *J. Am. Chem. Soc.*, 1912, xxxiv, 1706.

[5] Sherman, H. C., Kendall, E. C., and Clark, E. D., *J. Am. Chem. Soc.*, 1910, xxxii, 1083.

[6] McGuire, G., and Falk, K. G., *J. Gen. Physiol.*, 1919–20, ii, 217.

some of the series of sucrase actions, buffer mixtures were added directly to the enzyme-substrate mixtures. The indicators recommended by Clark and Lubs were used.[7]

Since a number of different procedures were employed to obtain the enzyme preparations from the bananas, these will be given in connection with the results obtained.

### *General Properties of Unripe and Ripe Bananas.*

Some of the general properties of the unripe and ripe bananas[8] will first be given. The pulp of the unripe banana adheres to the peel, making peeling difficult, while with the ripe banana the peel is easily removed. The pulp of the green banana is more fibrous in character and mashes with difficulty, in marked contrast to that of the ripe banana which readily forms a wet, soft paste. On mixing with water, the pulp of the former does not form a homogeneous mass, that of the latter does. In extracting (grinding) repeatedly with small portions of water and squeezing through muslin, a white cloudy liquid and a large quantity of insoluble residue are obtained from unripe banana pulp, while from ripe banana pulp most of the mixture can be squeezed through the muslin leaving only a small amount of very soft residue. The pulp of the unripe banana differs from that of the ripe banana also, in containing a sticky substance which discolors the hands.

The qualitative factors which have been given, as well as the color of the peel, serve to show the state of ripeness of the banana. More quantitative relations have been obtained by use of the "coefficient of ripeness" which represents the ratio of weight of pulp to weight of peel at the different stages.[3,4] This ratio increases in value as ripening proceeds.[1] The decrease in the percentage of starch present either in the peel or in the pulp might also be used as a quantitative measure of the state of ripeness.

[7] Clark, W. M., and Lubs, H. A., *J. Bact.*, 1917, ii, 1, 109, 191.

[8] We wish to thank the Fruit Dispatch Company for supplying the greater part of the bananas used in this investigation.

*Enzyme Tests with Unripe Bananas.*

The enzyme tests with unripe bananas can be summarized briefly; since (*a*) the general relations found were the same as with ripe bananas, except that (*b*) greater enzyme actions were obtained with ripe banana preparations than with unripe, and (*c*) the nature and properties of the unripe banana rendered working with it more difficult and less satisfactory.

The methods of studying the unripe banana pulp were as follows:

1. Pulp extracted with equal weight of water, filtered through muslin. Filtrate and residue (to which an equal quantity of water had been added) tested at different hydrogen ion concentrations in 1 per cent sucrose (also more concentrated) and starch solutions for 18 hours at 35°C.

2. Same as Method 1 with one-half the weight of water.

3. Same as Method 1 with one-quarter the weight of water kept between 5° and 10°C. during the preparation.

4. Pulp ground in food chopper, no water added.

5. Pulp and peel ground together in food chopper, no water added, and paste tested (mainly for amylase).

6. Pulp ground with twice its weight of 95 per cent ethyl alcohol, filtered through heavy muslin, and centrifuged. Residue ground with half its weight of water, filtered through muslin, centrifuged, and residue and liquid tested.

It may be pointed out here that upon the addition of Lintner starch solution to unripe banana extract, a precipitate was immediately formed. With ripe banana extracts there was no precipitate. With the unripe extract filtered through paper, upon the addition of Fehling's solution to determine the enzyme actions, a gelatinous precipitate was formed. Only small portions could therefore be used for the tests as otherwise filtration through the Gooch crucibles was difficult. No such difficulty was experienced with ripe banana extracts. Toluene was used as preservative throughout these experiments.

The general results of the enzyme tests were as follows: For amylase action, in no case were the results such as to show definitely the presence of this enzyme. In a few experiments small apparent

actions were observed, but, in view of possible experimental errors, these apparent actions were not of sufficient magnitude to prove conclusively the presence of such an enzyme. The object of the preliminary treatment with alcohol (Method 6) was for the purpose of dissolving possible inhibiting substances, such as tannin, with the simultaneous precipitation of the enzyme in order to obtain the latter separated from inactivating soluble material. The tests for sucrase were positive, considerable action being obtained. Since the ripe banana could be handled more readily, a more extended and quantitative examination of the sucrase from this source will be reported.

A few of the results with the unripe banana preparations are given to show the nature of the actions. 20 gm. of pulp obtained by Method 4 with 10 cc. of 2 per cent starch solution after 21 hours at 32°C. gave reducing substances corresponding to 5.0 mg. of $Cu_2O$ per gm. of pulp, and with 10 cc. of 20 per cent sucrose solution similarly, the reducing substances corresponding to 246 mg. of $Cu_2O$ per gm. of pulp. 20 gm. of pulp plus peel mixture obtained by Method 5 treated similarly gave with the starch no reducing substances, and with the sucrose reducing substances corresponding to 203 mg. of $Cu_2O$ per gm. of pulp plus peel.

The results of Bailey[4] on the action of air on the green banana pulp were confirmed, no amylase being obtained by this treatment.

*Soluble Sucrase Preparation from Ripe Bananas.*

Soluble and insoluble sucrase preparations were obtained from ripe bananas. As a result of a number of different methods of extraction, the following procedure was found to give the most satisfactory soluble preparation. The banana pulp was ground rapidly in a food chopper, the finest cutter being used, then mashed in a porcelain mortar with a wooden potato masher with normal sodium chloride solution (100 cc. for each 400 gm. of pulp). Toluene was added and the mixture then filtered through paper. The filtrate was dialyzed 18 to 24 hours in collodion bags against running water to remove the salt, soluble dialyzable carbohydrates, and other products. The resulting solution was used in the sucrase experiments.

A number of experiments were carried out in which the treated pulp was centrifuged and the supernatant liquid dialyzed and used, or where the treated pulp was squeezed through muslin and then centrifuged. The properties of the sucrase solutions so obtained were essentially the same, but the above procedure was the one finally adopted for studying the soluble banana sucrase.

The following methods of extraction did not yield preparations so satisfactory as the salt treatment described: Extraction with one-fourth weight or equal weight of water (fairly active preparations, in one case 2½ times as much action was obtained by the salt extraction as by the water extraction); extraction of small portions of banana with ten times the weight of water or salt solution; autolysis, followed by filtration, etc., as in the preparation of yeast sucrase[9] (slightly active filtrate obtained); and grinding in a ball mill for a long period of time (inactive filtrate).

It may be mentioned that repeated extractions did not offer any appreciable advantage over a single treatment. A small amount of active material could be obtained on a second extraction as compared with the first extract. Extracting for longer periods of time did not give appreciably more active solutions.

The following results were obtained in testing at different hydrogen ion concentrations. 10 cc. of the salt-extracted dialyzed solution plus 10 cc. of a 20 per cent sucrase solution were incubated for 2½ hours at 35°C. The actions are given in terms of mg. of $Cu_2O$ produced by the action of 1.0 cc. of original undialyzed filtrate corrected for blanks.

| pH | 3.0 | 3.5 | 4.0 | 4.5 | 5.0 | 5.5 | 6.0 | 6.5 |
|---|---|---|---|---|---|---|---|---|
| Actions | 183 | 481 | 489 | 478 | 458 | 375 | 135 | 52 |

Fig. 1 shows these results graphically. There is a zone of maximum action between pH 3.5 and 4.5, with rapid drops beginning between 3.5 and 3.0 and at 5.0. This optimum zone corresponds to that observed with yeast sucrase solutions, where the zone is of different widths in various experiments and under different conditions,[10] and with

[9] Nelson, J. M., and Born, S., *J. Am. Chem. Soc.*, 1914, xxxvi, 393.

[10] Sörensen, S. P. L., *Biochem. Z.*, 1909, xxi, 131. Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1911, xxxv, 386. Fales, H. A., and Nelson, J. M., *J. Am. Chem. Soc.*, 1915, xxxvii, 2769. Michaelis, L., and Rothstein, M., *Biochem. Z.*, 1920, cx, 217.

potato sucrase.[6] The exact optimum pH is difficult to determine in any case because of the flat portion of the curve, but with soluble

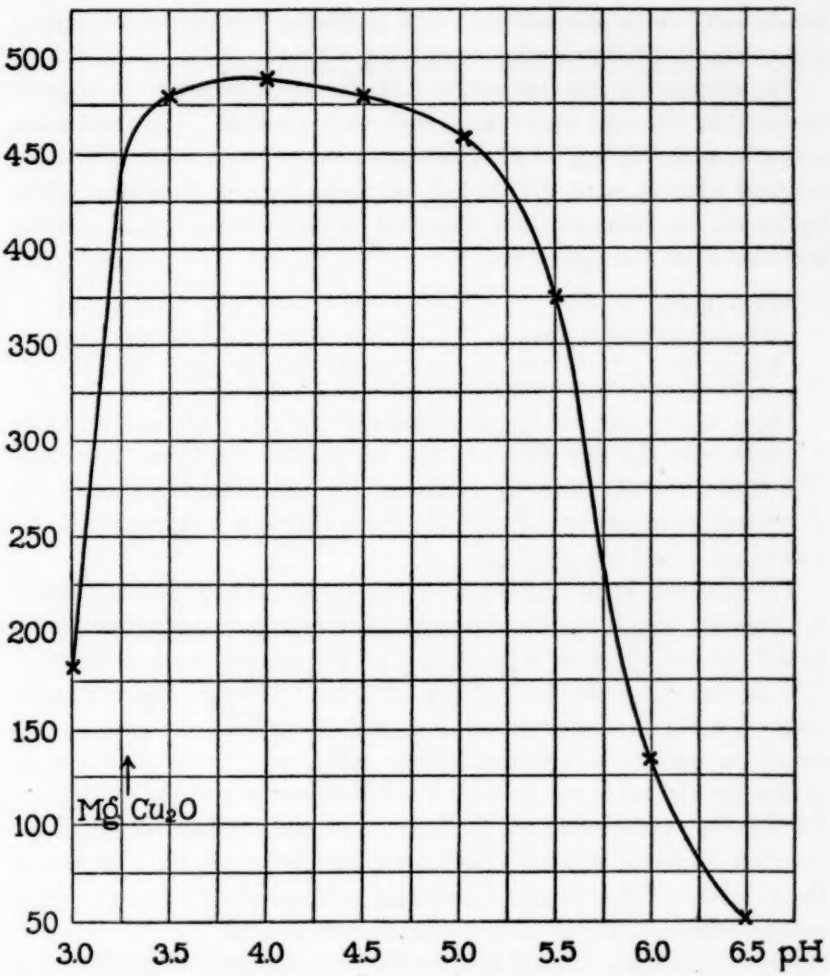


FIG. 1. Action of soluble banana sucrase preparation at different hydrogen ion concentrations on sucrose.

banana sucrase it is not far removed from 4.0 at 35°C. for 2½ hours action. The method of presenting these results is not the most satisfactory but it shows definitely the optimum conditions. Not

enough data were obtained to calculate the velocity constants satisfactorily, assuming that such constants would be obtained. In order to determine the different times for the same action, further assumptions would have to be introduced. The hydrogen ion concentrations of the solutions did not change during the actions.

The amounts of sucrase action with the same quantity of enzyme material at different intervals of time were studied. The results are given in terms of mg. of $Cu_2O$ obtained, calculated back to 1 cc. of original extract with 2.5 and 5 per cent sucrose solutions. The hydrogen ion concentration was that of the natural juice, not far removed from the optimum.

| Time (hrs.) | 1 | 2 | 3 | 4 | 5 | 6 | 24 |
|---|---|---|---|---|---|---|---|
| 2.5 per cent sucrose solution | 65 | 134 | 210 | 224 | 228 | 218 | 230 |
| 5.0 " " " " | 72 | 150 | 226 | 319 | 394 | 395 | 393 |

These results are shown graphically in Fig. 2. The one point at 6 hours with 2.5 per cent sucrose solution is evidently incorrect. The experimental errors are magnified by the calculations, since the difference indicated by 228 and 218 was caused by a difference in the weighings of 2.3 mg. of $Cu_2O$.

These results bring out the following facts. The action is a linear function of the time, the amounts hydrolyzed being proportional to the time of action until practically all the sucrose was hydrolyzed.[11] The action with 5 per cent sucrose solution was only slightly greater than with the 2.5 per cent solution, indicating that the sucrase was nearly saturated in the more dilute solution. However, the total action for the more concentrated solution was not twice as large as for the dilute solution in the limited times used. The products of the reaction evidently played a part here, interfering with the action of the sucrase. These results are similar to those obtained in the more extended investigations of yeast sucrase by others.

Active precipitates were obtained by the addition of alcohol or of acetone to the sucrase solution. These did not form clear solutions again, but showed considerable actions when tested as suspensions.

[11] Similar relations within certain limits have been observed with yeast sucrase. Nelson, J. M., and Vosburgh, W. C., *J. Am. Chem. Soc.*, 1917, xxxix, 790. Michaelis and Rothstein.[10]

Long continued treatment with alcohol appeared to inactivate the enzyme. These preparations were not studied further since the con-

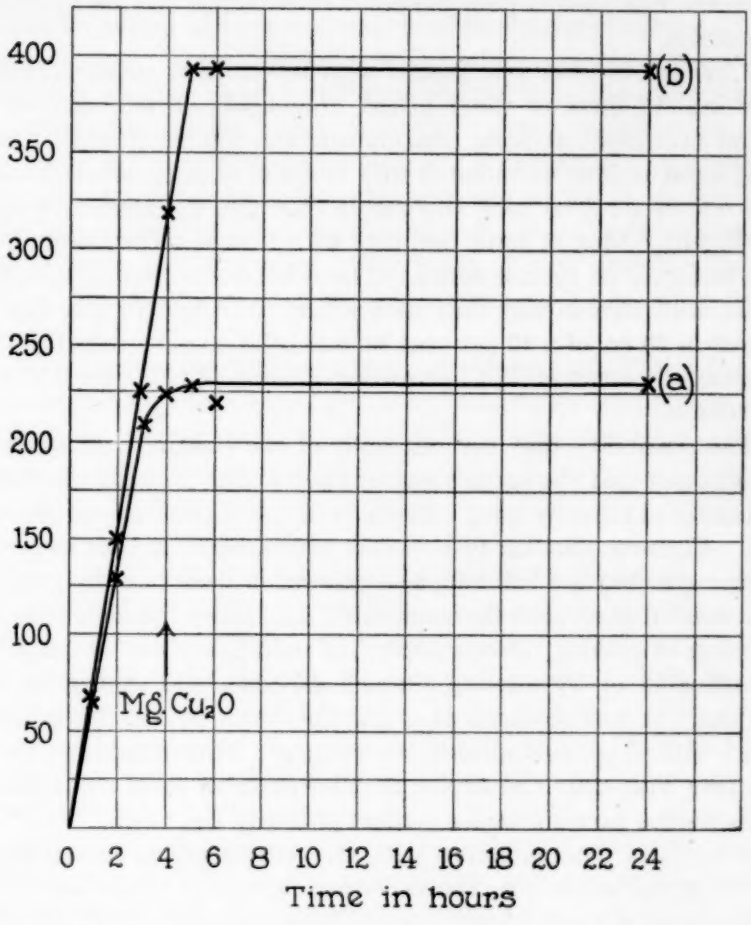


FIG. 2. Time-action curves of soluble sucrase preparation; (*a*) 2.5 per cent and (*b*) 5.0 per cent sucrose solutions.

version of soluble into insoluble sucrase preparation in a different way appeared to be of more direct interest.

*Insoluble Sucrase Preparation from Ripe Bananas.*

It was found that the water (or salt solution) insoluble residue from the ripe banana pulp possessed considerable sucrose-hydrolyzing action.[12]

2,760 gm. of pulp were ground with 700 cc. of N sodium chloride solution, 16 liters of water added, thoroughly stirred, allowed to stand over night at room temperature, and filtered through paper. The moist residue was treated with 10 liters of water similarly, and the residue (drained more thoroughly than the first residue) treated again with 6 liters of water and then with 8 liters. The filtrate from the last gave no sucrase action. The residue was dried by grinding twice with alcohol and then with ether. 0.75 gm. of this residue tested in 20 cc. of a 10 per cent sucrose solution gave reducing substances in 4 hours at 35°C. producing 355 mg. of $Cu_2O$ per 0.10 gm. of residue.

The possibility that the cell walls of the banana pulp were not broken and that the sucrase was retained within the cells was tested a number of times by using different methods of grinding and extracting. Vigorous grinding in a mortar with sand as well as long continued grinding in a ball mill, also followed in some cases by grinding in a mortar, gave, after thorough extraction, active insoluble residues. In place of filtering through paper, the residue was also obtained by centrifuging or by filtering through asbestos, and showed similar actions. It was obtained as a grayish brown powder by grinding twice with 95 per cent alcohol, separating by centrifuging, and finally washing with ether and drying on filter paper at room temperature. This residue gave a nitrogen content of nearly 1 per cent.

A series of determinations with the centrifuged moist residue at different hydrogen ion concentrations gave an optimum action between pH 4.0 and 4.5 with a rapid falling off beyond 6.0. In view of the character of the material, a more careful determination was not made. The results showed an optimum similar to that of the soluble sucrase preparation which was the main question involved.

[12] Euler and Svanberg (Euler, H., and Svanberg, O., *Z. physiol. Chem.*, cvii, 269) recently studied the sucrase actions of the residue from autolyzed and extracted yeast.

*Conversion of Soluble into Insoluble Sucrase Preparation.*

The finding of soluble and insoluble sucrase preparations or materials showing sucrase actions led to further developments. The optimum action for both was found to be at approximately the same pH (nearly 4.0). This raises the question, which was developed at some length in another connection,[13] as to whether a definite enzyme action was connected with a certain molecule or with a certain group which may be present in different molecules. If the latter view is adopted tentatively, the same active sucrase grouping would be present in the one case in a soluble molecular species, and in the other case in an insoluble molecular species. This point of view simplifies in some ways the consideration of the experiments to be described in this section.

That sucrase-carrying substances of different solubilities are present in banana pulp is shown by the fact that aqueous extraction yields one such, normal sodium chloride solution extraction following the aqueous extraction another, and the residue after repeated extraction a third. To judge from the hydrogen ion concentration for optimum action these sucrase actions are identical.

The most active and satisfactory soluble preparation was obtained by the salt extraction as described. The sodium chloride and the soluble simple sugars, etc., were removed by dialyzing over night in collodion bags against running tap water. The volume increased as a rule about 60 to 80 per cent. If the dialyzed sucrase solution was further dialyzed in a fresh collodion bag for 24 hours against tap water, the volume did not increase but a gel separated. The hydrogen ion concentration of the mixture did not change in the course of dialysis on the 2nd day. The gel could be filtered out readily by means of filter paper. The filtrate from the gel did not show sucrase action but the gel showed very marked activity. The gel was ground with alcohol twice and ether once and allowed to dry in the air at room temperature. It did not dissolve in water, but formed a gel with it. A suspension showed the following activity: 0.05 gm. of material in 20 cc. of a 10 per cent sucrose solution in 4 hours at 35°C. gave reducing substances producing 457 mg. of $Cu_2O$ per 0.01 gm. of

[13] Falk, K. G., The chemistry of enzyme actions, New York, 1921, 81.

solid preparation. It was therefore about fifteen times as active as the insoluble sucrase preparation obtained directly from the banana pulp. It showed a nitrogen content of 4.4 per cent; that is, about four times as large as that of the latter.

### *Amylase Results with Ripe Bananas.*

The amylase tests carried out with Lintner starch or with banana starch, in which the amounts of reducing sugars were determined, did not give definite evidence of the presence of a starch-splitting enzyme. These tests were carried out with extracts and suspensions prepared as described in the sucrase experiments. Before determining the amounts of reducing sugars formed, the mixtures were filtered through paper and an aliquot of the filtrate taken. Undialyzed extracts gave considerable blanks which increased on incubation because of the sucrase action on the sucrose contained in the banana. Dialyzed extracts gave little or no blanks. Isolated results at times showed an apparent action, but the results were not consistent enough or large enough to warrant the statement of a definite saccharogenic action on the starch. The mixtures were tested at different hydrogen ion concentrations and also in the presence of salt. The solid residue from the extractions behaved similarly.

In view of Bailey's positive results with the iodine test[4] (amyloclastic in place of saccharogenic actions) and since he found more marked actions with ripe than with unripe banana pulp, the reaction was studied in this way also. The method described by him was followed as closely as possible. It was found that, with the suspensions of banana pulp on incubation with Lintner starch, precipitates settled to the bottom of the tubes; that if these mixtures after incubation were tested with iodine after filtration, the filtrate gave no starch reaction but the precipitate on the paper became blue; that, if the whole mixture without filtering was shaken and tested with iodine, the precipitate was colored blue and settled leaving the supernatant liquid colorless. It is probable, though not altogether clear from the description of his experiments, that Bailey filtered or decanted these mixtures before testing with iodine. If that was the case, the amyloclastic actions which he described are open to ques-

tion.[14] Repeating his work, using the most satisfactory method of obtaining active preparations which he described, as well as extracts obtained as described with the sucrase experiments and incubating with 1 per cent Lintner starch solution for 24 to 40 hours at 30° to 35°C., it was found that no amyloclastic actions as marked as those described by Bailey were obtained. The greatest change which was observed in any of the tests was a change from the deep blue iodine starch reaction to a bluish violet color test. The amyloclastic actions may therefore be said to be extremely small if present at all. In all these tests, toluene was used as preservative.

## DISCUSSION.

The change of soluble sucrase material into insoluble during the simple treatment of dialysis raises the question of the state of the sucrase in the growing banana. On grinding to a pulp and extracting, soluble and insoluble preparations were obtained. This treatment may, however, have been sufficient to change materially the properties of the substances originally present. It is therefore conceivable that the sucrase may be present as a completely soluble substance in the ripening fruit and that the differences in solubility observed were due entirely to the treatment to which the fruit was subjected. It must also be recalled that toluene was added whenever the treatment extended over a greater period of time than a few minutes and that this toluene may exert a definite influence, possibly of a coagulating nature, upon the substances present.

This change in solubility was also observed when the bananas were ground in a ball mill with toluene present. 8 hours grinding followed by extraction gave a certain amount of soluble sucrase although less than was obtained by grinding in a mortar for a shorter period of time. Grinding for a week, however, resulted in the extract showing no sucrase action at all. The residue showed marked activity. It is probable that here, too, the soluble sucrase was converted into insoluble material. The reasons or causes for this change, whether due to dialysis and removal of certain products, the action of toluene, or other cause, are not known but will be investigated further.

[14] *Cf.* similar observations with potato amylase.[6]

The change of soluble into insoluble sucrase upon the simple treatments described illustrates the sensitive character of materials occurring in living matter. In this case, the enzyme property is not destroyed but a different property (solubility), which can be traced by following the enzyme action, is changed.

Sucrase has been generally taken to be one of the most hardy of the enzymes. It is not inactivated as rapidly as most other enzymes or under conditions which cause these to lose their activity completely. On the other hand, amylase has been found to be highly sensitive to outside influences and to be destroyed under comparatively simple conditions.[15] It is possible that the simple treatments, which changed the properties of the material carrying the sucrase action without destroying that action, may destroy the amylase action which would be expected to be present in the ripening banana. Such an explanation would account for the failure to obtain a definite and marked amylase action in banana pulp preparations or extracts.

The question of the conversion of starch into simpler carbohydrates may be considered further. For such an action in living, growing matter, the presence of the enzyme amylase, on the basis of past experience, is required. Experimental tests have not shown conclusively that this enzyme is present in the banana. Reasoning on the basis of the active amylase preparations described by Sherman[15] a minute quantity of such material would suffice to produce the changes observed in the banana, but, on the other hand, it should be possible to obtain experimental evidence of the presence of such an enzyme. It is also possible that substances are present in the banana which when brought into close contact, as by grinding, with the enzyme material, inhibit the action. It is possible to imagine a cellular structure of such nature that enzyme material and inactivating substance (possibly tannin) are separated in the fruit in the form and shape in which it occurs in nature and that artificial treatment involving destruction of the cell structure is accompanied or followed by profound changes in the cellular contents.

There is, however, another possible view-point. In ripening "The most conspicuous change is the long-recognized conversion of

[15] Sherman, H. C., and Schlesinger, M., *J. Am. Chem. Soc.*, 1915, xxxvii, 1305.

starch into sugars. It is most rapid while the fruits are turning from green to yellow. During this period the respiration rate increases manyfold, becoming greatest at the time when the rate of starch hydrolysis is most rapid."[2] Bailey[16] showed that the banana ripened in the presence of oxygen, but not in gases such as hydrogen, carbon dioxide, etc. It is therefore possible that the ripening process, or the breaking down of the starch, is not merely an amyloclastic action as this is commonly understood, but involves a simultaneous or preliminary oxidation reaction.

## SUMMARY.

A number of different methods of treatment of unripe and ripe bananas for the purpose of obtaining and studying sucrolytic and amylolytic enzymes are described.

No conclusive evidence of the presence of an amlyase could be obtained in any of the preparations.

The sucrase of unripe and ripe bananas was studied more extensively. With ripe bananas, both soluble and insoluble sucrase preparations were obtained. Conditions for converting the soluble into an insoluble form were found. The actions of the sucrase preparations as far as the hydrogen ion concentration for maximum action and the time-action relation are concerned are similar to the behavior of the yeast and the potato sucrase.

[16] Bailey, E. M., *J. Biol. Chem.*, 1905–06, i, 355.

# A THEORY OF INJURY AND RECOVERY.

## III. Repeated Exposures to Toxic Solutions.

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In previous papers a theory is developed which enables us to predict the behavior of *Laminaria* when transferred from sea water to toxic solutions and then replaced in sea water. If the theory is sound it should also enable us to predict the behavior of tissue transferred from one toxic solution to another. In order to put this to a test a variety of experiments was made in which the tissue was exposed to several solutions in succession.

The experiments and calculations were carried out as previously described.

### *I. Alternate Exposure to NaCl and Sea Water.*

The procedure may be illustrated by a typical experiment, the results of which are shown in Fig. 1.

The tissue was exposed for 20.8 minutes[1] to 0.278 M NaCl, during which time the resistance fell from 100 to 74.03 per cent. The tissue was then replaced in sea water. The resistance at any time, $T_R$, after this replacement may be calculated by means of a formula which has been explained in a previous paper.[2] The formula may be expressed as follows:

$$\text{Resistance} = (A+S)\left(\frac{K_A}{K_M-K_A}\right)\left(e^{-K_A T_R}-e^{-K_M T_R}\right)+Me^{-K_M T_R}+L\left(\frac{O}{90}\right)+10. \quad (1)$$

[1] This is corrected from 20 minutes (as explained in a previous paper[2]) in order to make it conform to the standard curve. An example of this is given in the present paper in discussing the change of resistance which occurs during the second exposure to NaCl.

[2] Osterhout, W. J. V., *J. Gen. Physiol.*, 1920–21, iii, 145.

in which $K_A = 0.0036$, $K_M = 0.1080$ and $M$ is equal to the observed resistance at the moment when the tissue is replaced in sea water less 10 (since the base line of the curve is taken as 10).

The value of $A$ may be obtained by the formula

$$A = 2700\, e^{-K_A T_E}. \tag{2}$$

in which $K_A = 0.018$ and $T_E$ is the time of exposure to NaCl (in this case 20.8 minutes).

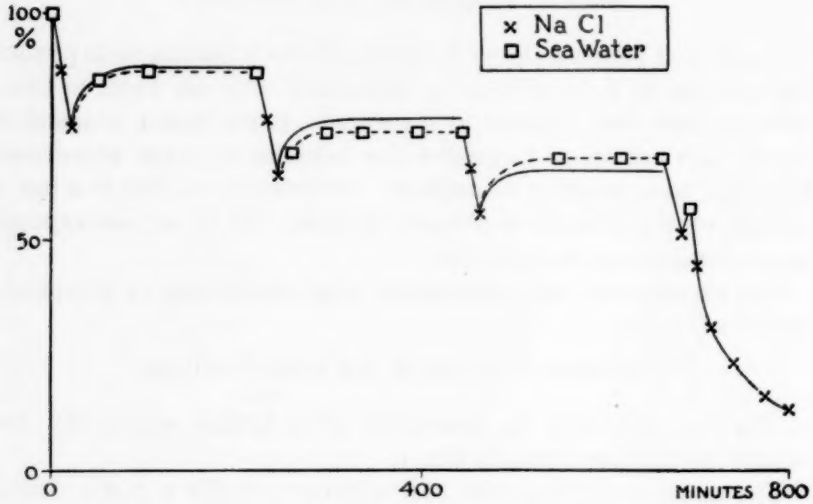


FIG. 1. Curves showing the electrical resistance of *Laminaria agardhii* in NaCl 0.52M and in sea water. Unbroken line, calculated values; broken line, observed values (at the extreme right these coincide). Average of ten or more experiments; probable error of the mean less than 10 per cent of the mean.

The value of $S$ may be obtained by the formula

$$S = 1041.77\left(\frac{K_R}{K_S - K_R}\right)\left(e^{-K_R T_E} - e^{-K_S T_E}\right) + 2.7\, e^{-K_S T_E} \tag{3}$$

in which $K_R = 0.04998$ and $K_S = 0.02856$.

The value of $O$ may be obtained by means of the formula

$$O = 89.1\left(\frac{K_N}{K_O - K_N}\right)\left(e^{-K_N T_E} - e^{-K_O T_E}\right) + 90\, e^{-K_O T_E} \tag{4}$$

in which $K_M = 0.03$ and $K_O = 0.0297$.

The value of $L$ may be obtained by means of the formula

$$L = 100 - \left[ 2700 \left( \frac{K_A}{K_M - K_A} \right) \left( e^{-K_A T_R} - e^{-K_M T_R} \right) + 90\, e^{-K_M T_R} + 10 \right] \quad (5)$$

in which $K_A = 0.0036$ and $K_M = 0.1080$ (these are the normal values in sea water).

After 20.8 minutes in the solution of NaCl the value of $T_E$ (in formulas (2), (3), and (4)) is 20.8 and the following results are obtained: $A = 1856.80$, $S = 484.06$, $M = 64.03$, $O = 88.41$. When the tissue has been replaced in sea water and left for 10.4 minutes[3] the value of $T_R$ (in formulas (1) and (5)) is 10.4 and the value of $L$ is found to be 1.33.

Substituting these values in formula (1) we find that when the tissue has been replaced in sea water the resistance at the end of 10.4 minutes is 83.49. Proceeding in this manner we calculate the resistance at various intervals after replacement in sea water and obtain the first (calculated) recovery curve shown in Fig. 1. It is evident that it is in fairly good agreement with the observed values.

After 200 minutes in sea water (during which the resistance rose to 87.10 per cent and remained practically constant) the tissue was replaced in the solution of NaCl. In the course of 21.2 minutes[4] the resistance fell from 87.10 to 64.18. It was then replaced in sea water. The recovery curve may be calculated as before, the only differences being as follows:

1. On replacing the tissue in sea water the destruction of $O$ (by the reactions $N \rightarrow O \rightarrow P$) ceases (or becomes negligible); hence the value of $O$ at the beginning of the second exposure (if equilibrium has been reached) is that of the observed resistance less 10, or $87.10 - 10 = 77.10$. We find by means of formula (4) that when $O$ at the start equals 90 it loses 11.95 during an exposure of 21.2 minutes to the solution of NaCl, but as it only equals 77.10 at the start the loss will be $11.95\ (77.10 \div 90) = 10.23$. Subtracting this from 77.10 gives 66.87, the value of $O$ at the end of the second exposure, and adding 10 (since the base line is 10) makes 76.87, the level to which the resistance should rise after the second exposure.

[3] This is corrected from 10 minutes[1] as explained in a former paper.

[4] The actual time was 20 minutes: the manner in which the corrected figure is obtained is explained in a subsequent paragraph.[1]

2. At the start of the first recovery[5] $S$ is rapidly converted into $A$ but is partially restored during the subsequent stay in sea water and at the beginning of the second exposure equals 2.7 $(O \div 90)$ in which $O$ has the value given above (77.10).

3. During exposure to NaCl the value of $R$ diminishes from $R_0$ to $R_1$ according to the formula

$$R_1 = R_0 e^{-K_R T_E} = 1041.77\, e^{-(0.0498)42} \qquad (6)$$

in which $R_0$ = the value of $R$ before the first exposure (1041.77) and $T_E$ equals the total exposure to NaCl (20.8 + 21.2 = 42).

It is evident that unless $R$ is restored during the period in sea water the speed of recovery will fall off somewhat with each successive exposure.

4. The value of $M$ is the observed resistance (at the end of the second exposure) less 10 or 64.18 − 10 = 54.18.

5. The value of $A$ is obtained by multiplying by 30 the resistance observed at equilibrium (less 10). This is based upon the following considerations:

Just before the beginning of the second exposure $A$ and $M$ are assumed to be in equilibrium in sea water, in which case as much of $A$ must decompose in any minute as of $M$ (otherwise $M$ would not remain constant). But the amount of $A$ which decomposes in 1 minute is $AK_A$ and of $M$ is $MK_M$; and since $K_M$ is 30 times as great as $K_A$ it follows that $A = 30M$. At the beginning of the second exposure $M = 87.10 - 10 = 77.10$ and $A = (77.10)30 = 2313$.

In order to ascertain how the resistance would change during the second exposure if it conformed to the standard curve employed in a previous paper[2] we may employ the formula

$$\text{Resistance} = 2313\left(\frac{K_A}{K_M - K_A}\right)\left(e^{-K_A T_E} - e^{-K_M T_E}\right) + 77.1\, e^{-K_M T_E} + 10 \qquad (7)$$

in which $K_A = 0.018$, $K_M = 0.540$ and $T_E$ = time the tissue has remained in the solution of NaCl. Comparing the values thus obtained with the observed resistance after an exposure of 20 minutes

[5] If the value of $O$ were 90, $S$ would be completely restored to its original value of 2.7 but since $O$ has fallen to 77.10 it can only restore $S$ to 2.7 $(77.10 \div 90)$.

we find that if the time is multiplied by 1.06 (making it 21.2 minutes) the observed resistance (64.18) agrees with the standard curve. This figure is therefore adopted. The value of $T_E$ in formulas (2), (3) and (4) should now correspond to the total exposure to NaCl, and is 20.8 + 21.2 = 42.

These data were employed in calculating the second recovery curve and the results are shown in Fig. 1. The third recovery curve was calculated in the same fashion.

Instead of waiting for the establishment of equilibrium we may replace the tissue in NaCl after it has been for a short time in sea water. During the fourth recovery, after the tissue had been 10.2 minutes in sea water and the resistance had risen to 54.92 per cent it was replaced in sea water: the subsequent fall in resistance was calculated by means of formula (7). For the value 77.1 in this formula we must substitute the observed resistance less 10, or 55.89 − 10 = 45.89; and in place of 2313 we must substitute the present value of $A$. We assume that at the beginning of the fourth exposure to NaCl equilibrium had been reached in sea water: hence as the resistance was 68.10 the value of $A$ (which we may call $A_1$) is, $A_1 = 30(68.10 - 10)$. During the fourth exposure to NaCl (lasting 20.4 minutes) the value of $A_1$ diminished to $A_2$ according to the formula

$$A_1 e^{-(0.018)20.4} = A_2$$

On replacing the tissue in sea water $A_2$ was augmented by the conversion of $S$ into $A$. The value of $S$ is found according to formula (3) in which $T_E$ is equal to the total time of exposure (20.8 + 21.2 + 20.8 + 20.4 = 83.2). We may call this $S_1$. Hence the value of $A$ immediately after replacement in sea water is $A_3 = A_2 + S_1$. During the subsequent 10.2 minutes in sea water $A_3$ diminished to $A_4$ according to the formula

$$A_3 e^{-(0.0036)10.2} = A_4$$

But at the same time it received an addition from the decomposition of $O$; the amount of this may be found as follows: The loss of $A$ in sea water under normal conditions[6] in 10.2 minutes is

[6] The principle upon which this formula is based is explained in a previous paper in discussing the loss of $M$ and its replacement by $O$. In the present case the effect of $S$ is negligible since the amount of $S$ in sea water is only 2.7.

$$\text{Loss} = 2700 - \left( 2700e^{-(0.0036)10.2} \right) = 97.26 \qquad (8)$$

and this could be completely replaced by $O$ if $O$ were intact. But since $O$ has diminished[7] from 90 to 50.86 it can supply only $97.26(50.86 \div 90) = 54.95$. This must be added to $A$ giving $A_5 = A_4 + 54.95$. The value of $A_5$ must be substituted for 2313 in formula (7). This enables us to calculate the fall of resistance after the last recovery (of 10.2 minutes). Fig. 1 shows the values so obtained and also the observed values.

### *II. Alternate Exposure to $CaCl_2$ and Sea Water.*

When the tissue of *Laminaria* is transferred from sea water to a solution of $CaCl_2$ (of the same conductivity as sea water) the resistance rises and then falls as shown in Fig. 2. When it is replaced in sea water the resistance falls (much more rapidly than if left in the solution of $CaCl_2$) and eventually becomes stationary. This fall of resistance may be spoken of as recovery, since it may be regarded as analagous to the rise of resistance which occurs when tissue is transferred from NaCl to sea water.

Recovery after exposure to $CaCl_2$ may be calculated in precisely the same manner as recovery after exposure to NaCl. The only difference is that in formulas (2), (3), (4), (6), and (7) we must employ for the velocity constants ($K_N$, $K_O$, $K_R$, $K_S$, $K_A$ and $K_M$) the values given for $CaCl_2$ in Table II of the preceding paper.[8] In formulas (1) and (5) the values of the velocity constants are always the same ($K_A = 0.0036$ and $K_M = 0.1080$) since these are the values which are normal for sea water.

Results of such calculations are shown in Fig. 2 together with the observed values.

[7] This is calculated as follows: At the beginning of the fourth exposure $O = 68.10 - 10 = 58.10$. If its value were 90 it would lose 11.23 during an exposure of 20.4 minutes to NaCl. Since $O = 58.10$ the loss will be $11.23\ (58.10 \div 90) = 7.24$: subtracting this from 58.10 we have 50.86.

[8] Osterhout, W. J. V., *J. Gen. Physiol.*, 1920–21, iii, 415.

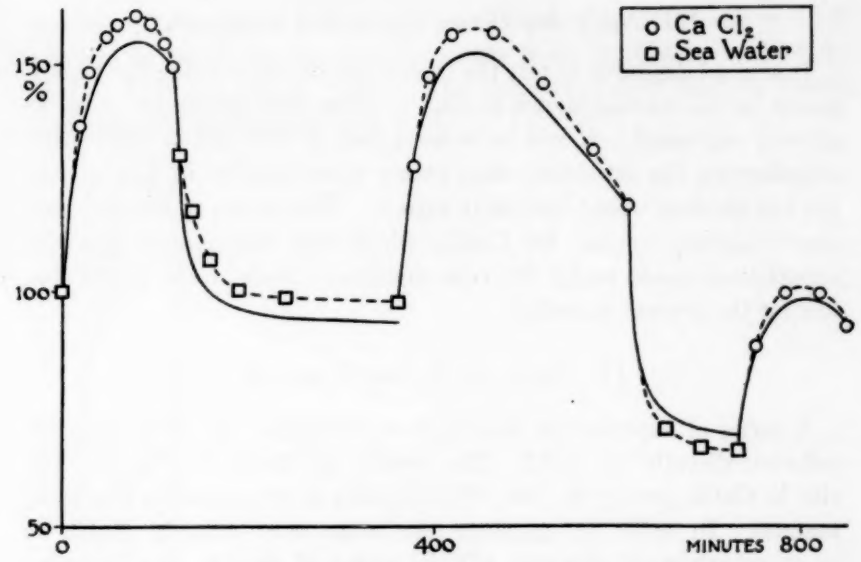


FIG. 2. Curves showing the electrical resistance of *Laminaria agardhii* in $CaCl_2$ 0.278M and in sea water. Unbroken line, calculated values; broken line, observed values. Average of ten or more experiments; probable error of the mean less than 10 per cent of the mean.

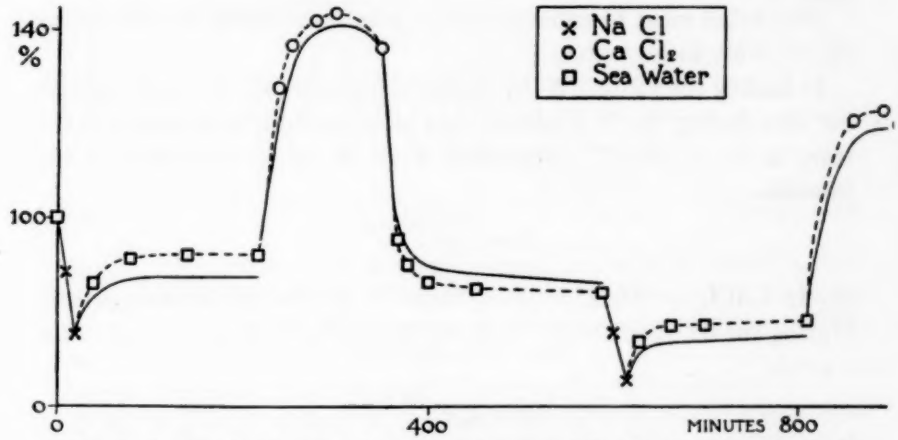


FIG. 3. Curves showing the electrical resistance of *Laminaria agardhii* in NaCl 0.52M, in $CaCl_2$ 0.278M and in sea water. Unbroken line, calculated values; broken line, observed values. Average of ten or more experiments; probable error of the mean less than 10 per cent of the mean.

### *III. NaCl, Sea Water, $CaCl_2$, Sea Water, etc.*

It seemed desirable to test the theory further by varying the experiments in the manner shown in Fig. 3. The calculations are made as already explained. It will be noticed that in this and in some other experiments the resistance rises rather more rapidly in $CaCl_2$ than the calculations would lead us to expect. This is due to the fact that the "standard curve" for $CaCl_2$, which was based upon previous experiments made under different conditions, seems to be a little too low for the present material.

### *IV. $CaCl_2$, NaCl, Sea Water, etc.*

A series of experiments was made to determine the effect of $CaCl_2$ followed directly by NaCl. The results are shown in Fig. 4. The rise in $CaCl_2$ during the first 91.8 minutes is calculated in the usual manner. In order to calculate the subsequent drop in NaCl we must substitute in formula (7) the value of $M$; i.e., the observed resistance (less 10) at the beginning of exposure to NaCl. In place of 2313 we must substitute the value of $A$, which is $A_1 = 2700e^{-(0.0018)91.8}$.

During the exposure of 60.6 minutes to NaCl the value of $A$ changes from $A_1$ to $A_2 = A_1e^{-(0.018)60.6}$.

This value must be substituted for $A$ in Formula (1) in calculating the recovery in sea water.

In finding the value of $S$ (by means of formula (3)) we must remember that during the 91.8 minutes in $CaCl_2$ the value of $R$ (which at the start is $R_0 = 1041.77$) diminishes from $R_0$ to $R_1$ according to the formula

$$R_1 = R_0 e^{-91.8 K_R}$$

$K_R$ in $CaCl_2$ = 0.012532 (See Table II of the preceding paper[3]). During the 60.6 minutes in NaCl $R_1$ diminishes to $R_2$ according to the formula

$$R_2 = R_1 e^{-60.6 K_R}$$

$K_R$ in NaCl = 0.04998.

We must also bear in mind that $O$ diminishes during the exposure. Since this process is 6 times as rapid in NaCl as in $CaCl_2$ we may

consider 91.8 minutes in $CaCl_2$ to be equivalent to 91.8 ÷ 6 = 15.3 minutes in NaCl and the total exposure to be equivalent to 60.6 + 15.3 = 75.9 minutes in NaCl.[9] The value of *O* may then be found by means of formula (4).

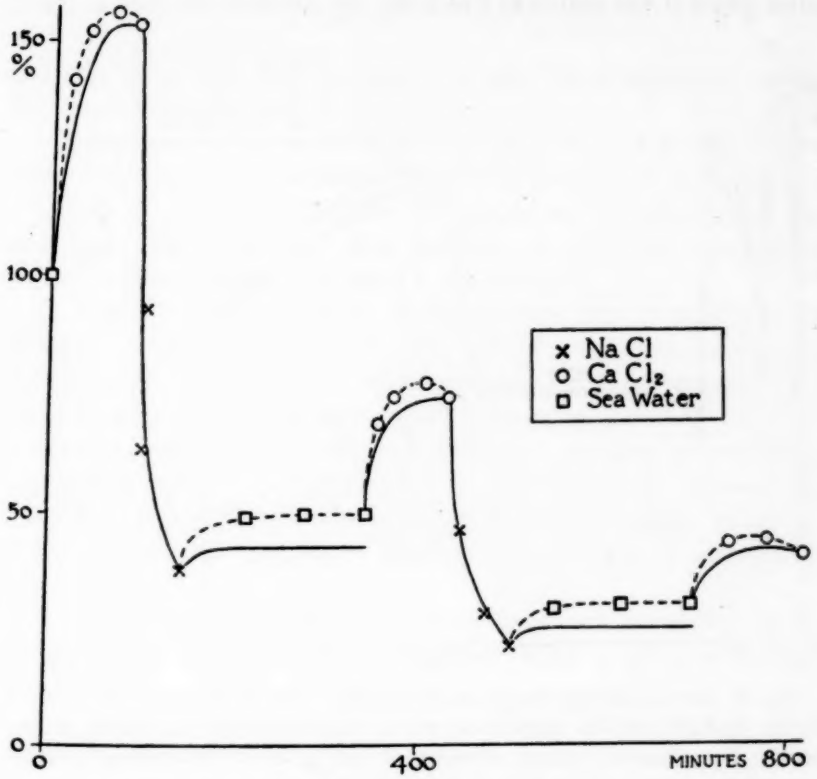


FIG. 4. Curves showing the electrical resistance of *Laminaria agardhii* in NaCl 0.52M, in $CaCl_2$ 0.278M and in sea water. Unbroken line, calculated values; broken line, observed values. Average of ten or more experiments; probable error of the mean less than 10 per cent of the mean.

[9] This involves the assumption that *O* is not restored to any extent during recovery in sea water. This assumption may not be quite correct, especially at the start, but even in that case the present calculation would not be appreciably altered.

### *V. $CaCl_2$, NaCl, $CaCl_2$, NaCl, Sea Water, etc.*

A series of experiments was performed in which tissue was placed in $CaCl_2$ for 30 minutes, then in NaCl for 10 minutes, then in $CaCl_2$ for 60 minutes. The tissue was allowed to recover in sea water, after which it was placed in $CaCl_2$ for 360 minutes, and then in NaCl.

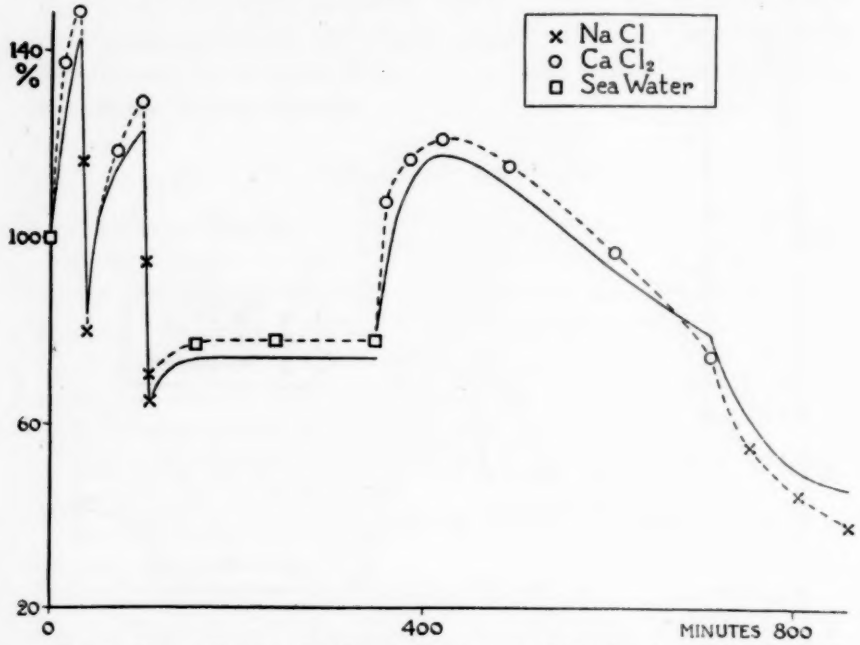


FIG. 5. Curves showing the electrical resistance of *Laminaria agardhii* in NaCl 0.52M, in $CaCl_2$ 0.278M and in sea water. Unbroken line, calculated values; broken line, observed values. Average of ten or more experiments; probable error of the mean less than 10 per cent of the mean.

In this case the observed time was not corrected (i.e., was not multiplied by a factor) as in the previous calculations. In consequence the calculated and observed values do not correspond at the beginning of each exposure, the only exception being after recovery in sea water, in which case it was assumed[10] that equilibrium had been

[10] In this case the tissue did not remain long enough in sea water to establish equilibrium but it was so nearly established that only a very small error is involved in regarding it as complete. In cases where it is not completely established the final equilibrium may be approximated with sufficient accuracy by extrapolation.

reached and that in consequence $A_0 = 30M$ (the value of $M$ being that of the observed resistance less 10). This value of $A$ was taken for the subsequent calculations. During the subsequent exposure to $CaCl_2$ $A_0$ diminished to $A_1$ according to the formula

$$A_1 = A_0 e^{-(0.0018)360}$$

and this value was used in calculating the fall of resistance during the final exposure to NaCl.

Experiments similar to those shown in Figs. 1, 2, 3, 4, and 5 have been made, in which mixtures of NaCl + $CaCl_2$ have been used in a variety of ways. In this case we employ for the calculations the constants appropriate for each mixture, as given in a preceding paper.[8] In general the agreement is satisfactory.

It should be noted that we do not employ new constants to fit these curves but that in every case we use the constants already determined as the result of other and quite different experiments. In view of this the results have a special significance.

These experiments, and those described in previous papers, seem to afford a sufficient test of the theory. The agreement between the calculated and observed values appears to be satisfactory whenever a sufficiently large number of readings are averaged in arriving at the observed values.

It is evident that the equations which have been developed enable us to predict the behavior of the tissue under a great variety of conditions.

The mechanism which has been postulated in developing these equations consists essentially of a series of catenary reactions. There can be no doubt that catenary reactions play a large part in life phenomena and it would seem that the rôle assigned to them in the present discussion involves no unreasonable assumption.

It may be desirable to call attention to features of this mechanism which are of general interest from a theoretical viewpoint. It is evident that by means of a simple catenary system we can account for practically all the processes which occur in the organism. If such a system is present in the egg we can easily picture all of the subsequent development as due to this system, without the intro-

duction of any new reactions. All that we need to postulate is that during development the relative rates of the reactions change. The processes involved in irritability, as well as those concerned in injury and death, may be accounted for in this way. We thus arrive at a very simple conception of the underlying mechanism of life processes, which may be useful in formulating a theory of living matter.

As an illustration of the effect of changes in relative rates we may take the substance, $M$, in the series

$$O \rightarrow S \rightarrow A \rightarrow M \rightarrow B.$$

It is evident that we may increase the value of $M$ in a variety of ways, as by increasing the rate of $O \rightarrow S$, $S \rightarrow A$, $A \rightarrow M$, or of any two of these reactions, or of all of them simultaneously. We may decrease the amount of $M$ by decreasing the rates of these reactions or by increasing the rate of $M \rightarrow B$ (or increase $M$ by decreasing this rate). We may likewise increase (or decrease) $M$ by increasing (or decreasing) the amount of $O$. Furthermore if side reactions occur, such as $N \rightarrow O \rightarrow P$ or $R \rightarrow S \rightarrow T$ their rates will also affect the amount of $M$. It must also be remembered that any one of the substances in the system might act as an accelerator or inhibitor of any of the reactions. With such a system a great variety of processes is possible.

It is possible that such systems may play an important rôle in the fundamental processes of living matter.

## SUMMARY.

Tissues of *Laminaria* transferred from sea water to solutions of pure salts, and thence to other solutions of pure salts, or to sea water, behave in a manner which can be predicted by means of the equations previously developed.

The behavior of the tissue may be explained as due to a series of catenary reactions. It is possible that a similar explanation may be applied to other fundamental life processes.

# THE RATE OF GROWTH OF THE DAIRY COW.

## Extrauterine Growth in Weight.

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If a cell of the body of an animal is divided into two equal daughter cells, then clearly each daughter cell contains only half the chromatin contained in the mother cell. Before dividing again, however, the chromatin in each daughter cell is increased to the amount contained in the mother cell at the expense of the surrounding cytoplasm. This process of division and increase of chromatin is continued until a definite equilibrium ratio is reached between the cytoplasm and chromatin, when further division ceases, unless the cytoplasm continues to increase by further absorption of food. These facts, found by Sachs, Morgan, Driesch, Boveri, and others[1] and the further fact that the increase of chromatin at the expense of cytoplasm seems to be a chemical process, as seen for example by the dependence of the increase of chromatin on the presence of oxygen,[1] led Loeb[1,2] to advance the theory that the process of cell division is ultimately controlled by the fundamental chemical laws of mass action, and equilibrium. He also concluded from the fact that the cells multiply in a geometrical progression that the synthesis of chromatin is limited by an autocatalytic monomolecular reaction; that is, one which is accelerated by its own product.

What was said of the growth of chromatin must also hold true for the growth of the cell in the presence of food, and for the growth of any tissue and of the whole body of a metazoan; since the growth of the cell is in geometric progression, the growth of a tissue is the

[1] Cf. Loeb, J., The dynamics of living matter, New York, 1906, 58–66.

[2] Loeb, J., *Biochem. Z.*, 1906, i, 34; *Biol. Centr.*, 1910, xxx, 347.

resultant of multiplication of cells, and the growth of the whole body is the resultant of the interdependent and harmonious growth of the various tissues. The whole body may therefore be said to be determined by a limiting autocatalytic reaction and as such the rate of growth of the mass of the body should follow the velocity equation of an autocatalytic monomolecular reaction. Robertson[3] and Ostwald[4] simultaneously but independently showed this to be the case.

The velocity equation of an autocatalytic monomolecular reaction may be derived as follows: Let $A$ be the amount of limiting growth substance at the beginning of growth which is ultimately destined in the presence of nutrients to be converted into tissue; let the amount

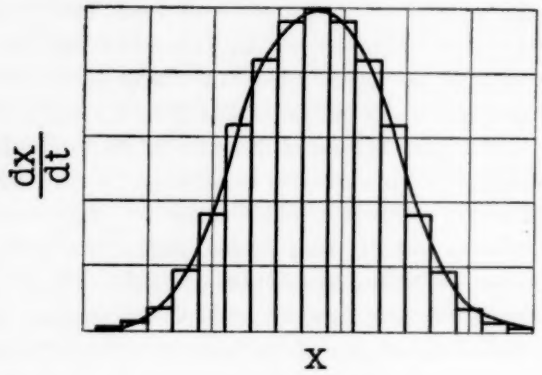


FIG. 1. Curve of $\frac{dx}{dt} = x(A - x)$. Ordinates represent velocity, $\frac{dx}{dt}$; abscissæ represent ascending values of $x$.

so converted be $x$; and, therefore, $(A - x)$ remains unconverted. By hypothesis, the velocity of growth $\frac{dx}{dt}$ is proportional to both $(A - x)$ and also to $x$ which acts as the catalyzer of the reaction, that is,

$$\frac{dx}{dt} = Kx(A - x) \qquad (1)$$

[3] Robertson, T. B., *Arch. Entwicklungsmechn. Organ.*, 1907–08, xxv, 581; *Biol. Centr.*, 1910, xxx, 316; Principles of biochemistry, for students of medicine, agriculture and related sciences, Philadelphia, 1920.

[4] Ostwald, W., Vorträge und Aufsätze über Entwicklungsmechanik der Organismen, Leipsic, 1908, v, cited by Loeb (1910),[2] and Robertson (1910).[3]

where $K$ is the velocity constant. On plotting $\frac{dx}{dt}$ for various values of $x$ a curve of a rising and falling type is obtained with a maximum at the center where $x = A - x$ or $x = \frac{1}{2}A$ as shown in Fig. 1.

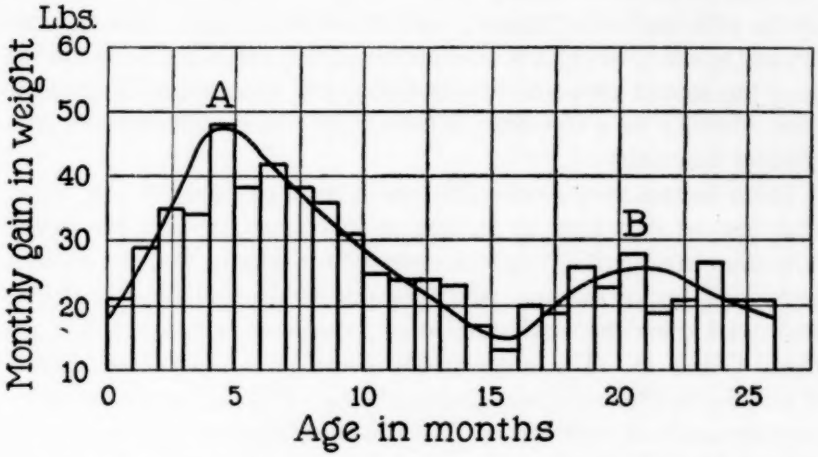


FIG. 2. Velocity curve of growth of the Jersey cow. Ordinates represent velocity of growth, the height of the rectangles representing monthly gains in weight; abscissæ represent age in months.

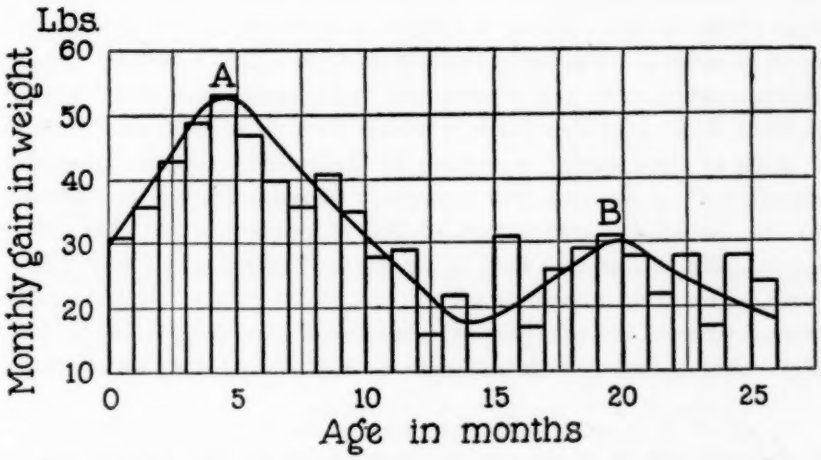


FIG. 3. Velocity curve of growth of the Holstein cow. Ordinates and abscissæ have the same meaning as in Fig. 2.

A comparison of Fig. 1, the theoretical velocity curve of an autocatalytic monomolecular reaction, with Figs. 2 and 3, the velocity curves of growth of a group of dairy cows in this Station,[5] shows obvious similarities.

These postuterine velocity curves of growth show two reactions or cycles with maxima at about 5 and 20 months of age. The assymetry of the curve of cycle A may be tentatively explained by assuming that the second cycle, B, begins before the completion of cycle A, thus resulting in a superimposition of the two cycles with the consequent assymetry.

Other factors may conceivably enter, such as deposition of fat at that age, as suggested by Robertson in connection with his work.[3] The other irregularities might be expected considering that the animals were kept under average, which means highly variable, conditions and considering the small number of animals on which the data are based (Table I). The irregularities in the second extrauterine cycle B may be further accounted for by the fact that for reasons of economy the animals were bred at 20 to 21 months of age, calving at the ages of 29 to 30 months.

Cycle B is undoubtedly the last growth cycle, comparatively little growth being made after the age of 30 months. According to Donaldson[6] and Robertson,[3] there are at least three such cycles in the mammal; if the tricyclic theory is true, then there should be at least one cycle *in utero* in addition to the extrauterine cycles A and B. There is some evidence in this department, not enough, however, for publication, indicating that there is indeed such an intrauterine cycle.

Each of these cycles, according to Robertson,[3] should follow the equation of an autocatalytic reaction. It is interesting, in addition to the qualitative comparison of the experimental and theoretical curves, also to find out what is the approximate quantitative agreement between the experimental and calculated values. Equation (1) cannot be used for this purpose since, while the weights of the animals were recorded with reference to time, their age, the velocity

[5] Eckles, C. H., *Univ. Missouri Agric. Exp. Station, Research Bull. 36*, 1920.

[6] Donaldson, H. H., Boas memorial volume, New York, 1906, cited by Robertson (1907–08).[3]

**TABLE I.**

*Data on the Growth of the Dairy Cow.**

| | Holstein cows. | | | | | Jersey cows. | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Age. | Number of animals weighed. | Average weight. | Range in weight.† | Average deviation from average weight.‡ | Deviation.§ | Number of animals weighed. | Average weight. | Range in weight. | Average deviation from average weight. | Deviation. |
| *mos.* | | *lbs.* | *lbs.* | *lbs.* | *per cent* | | *lbs.* | *lbs.* | *lbs.* | *per cent* |
| 0 birth | 83 | 90 | 57 | | | 94 | 55 | 35 | | |
| 1 | 9 | 121 | 23 | 4.3 | 3.6 | 6 | 76 | 31 | 10.7 | 14.0 |
| 2 | 9 | 157 | 22 | 5.6 | 3.5 | 7 | 105 | 34 | 11.0 | 10.5 |
| 3 | 9 | 200 | 48 | 13.1 | 6.6 | 7 | 140 | 54 | 14.4 | 10.3 |
| 4 | 9 | 249 | 66 | 15.3 | 6.2 | 8 | 174 | 73 | 18.5 | 10.6 |
| 5 | 9 | 302 | 97 | 20.2 | 6.7 | 9 | 222 | 83 | 22.0 | 9.9 |
| 6 | 9 | 349 | 129 | 21.9 | 6.3 | 10 | 260 | 101 | 23.7 | 9.1 |
| 7 | 11 | 389 | 169 | 34.0 | 8.8 | 10 | 302 | 107 | 20.8 | 6.9 |
| 8 | 11 | 425 | 217 | 31.5 | 7.4 | 10 | 340 | 134 | 22.7 | 6.7 |
| 9 | 11 | 466 | 251 | 41.8 | 8.95 | 11 | 376 | 152 | 23.5 | 6.3 |
| 10 | 11 | 501 | 268 | 50.5 | 10.0 | 11 | 407 | 146 | 23.6 | 5.8 |
| 11 | 11 | 529 | 291 | 35.7 | 6.7 | 11 | 432 | 134 | 35.2 | 8.0 |
| 12 | 11 | 558 | 276 | 39.9 | 7.2 | 11 | 456 | 124 | 35.9 | 7.9 |
| 13 | 11 | 574 | 237 | 36.3 | 6.3 | 11 | 480 | 118 | 34.3 | 7.1 |
| 14 | 11 | 596 | 243 | 38.6 | 6.4 | 11 | 503 | 127 | 25.8 | 5.1 |
| 15 | 12 | 612 | 186 | 42.9 | 7.0 | 11 | 520 | 114 | 31.8 | 6.1 |
| 16 | 13 | 643 | 164 | 42.2 | 6.6 | 11 | 533 | 87 | 27.4 | 5.1 |
| 17 | 14 | 660 | 174 | 45.7 | 6.9 | 12 | 553 | 95 | 29.3 | 5.3 |
| 18 | 14 | 686 | 162 | 36.0 | 5.3 | 13 | 572 | 78 | 22.4 | 3.9 |
| 19 | 14 | 715 | 183 | 33.0 | 4.6 | 13 | 598 | 126 | 31.4 | 5.2 |
| 20 | 14 | 746 | 123 | 31.9 | 4.3 | 13 | 621 | 130 | 32.6 | 5.2 |
| 21 | 14 | 774 | 216 | 47.0 | 6.0 | 13 | 649 | 151 | 32.6 | 5.0 |
| 22 | 14 | 796 | 223 | 51.5 | 6.5 | 13 | 668 | 108 | 33.3 | 5.0 |
| 23 | 14 | 824 | 177 | 43.1 | 5.2 | 12 | 689 | 179 | 47.6 | 6.9 |
| 24 | 14 | 841 | 151 | 38.8 | 4.6 | 12 | 716 | 133 | 40.2 | 5.6 |
| 25 | 14 | 869 | 188 | 48.6 | 5.6 | 12 | 737 | 147 | 39.3 | 5.3 |
| 26 | 14 | 893 | 234 | 42.0 | 4.7 | | 758 | 188 | | |
| 27 | 14 | 925 | 261 | 54.5 | 5.9 | | 770 | 212 | | |
| 28 | 14 | 966 | 253 | 55.0 | 5.7 | | 784 | 92 | | |
| 29 | 13 | 994 | 255 | 58.7 | 5.9 | | 804 | | | |

* The animals were bred at 20 to 21 months of age, calving at 29 to 30 months of age.

† Range = difference between observed extremes; thus the observed extreme weights of Holstein calves were 55 and 112 pounds, a range of 57 pounds.

‡ Average deviation from average weight = $a.d. = \frac{\Sigma d}{n}$, a numerical measure of the amount by which a new observation taken under the given conditions is likely to differ from the average value.

§ Percentage deviation = $\frac{a.d.}{m} \times 100 = \frac{\text{Average deviation}}{\text{Average weight}} \times 100.$

$\frac{dx}{dt}$ in equation (1) is not expressed as a function of time. Equation (1) is therefore first integrated. The integration is performed by writing

$$\frac{dx}{dt} = Kx(A - x)$$

in the form

$$dt = \frac{dx}{Kx(A - x)}$$

which is a standard form ready for integration

$$\int dt = \int \frac{dx}{Kx(A - x)}$$

Integrating and putting $K$ for $KA$ we get

$$t = \frac{1}{K} \log \frac{x}{A - x} + C$$

or

$$\log \frac{x}{A - x} = Kt + C$$

where $C$ is the integration constant. The precise meaning of $C$, the integration constant, and $t$, time, may be obtained as follows: Let $t$, time, be counted from the maximum of the velocity curve (A in the figure); that is, where $x = A - x$. The value of $t$ at the maximum point is therefore zero, so is $\log \frac{x}{A - x}$, and $C$ also, and the equation is simplified to

$$\log \frac{x}{A - x} = Kt$$

where $t$ is the time interval between the maximum point of growth and any other time on the curve. Instead of $t$, we may write $(t - t_1)$ when $t_1$ is the age of the animal at the maximum point of growth, and $t$ is any age. The equation becomes

$$\log \frac{x}{A - x} = K(t - t_1) \qquad (2)$$

where $x$, the weight of the animal, is expressed as a function of its age, $t$. Equation (2) is the form used by Robertson.[3] If desired, equation (2), a weight-age equation, may be changed to a velocity-age equation by differentiating it after some simplification and obtaining[7]

$$\frac{dx}{dt} = \frac{Ke^{Kt}}{(1+e^{Kt})^2}$$

where $\frac{dx}{dt}$ is the velocity, $t$ and $K$ have the same significance as in equation (2), $e = 2.718$, the base of natural logarithms. Equation (2) will, however, be used on account of the relative simplicity of its application to the data.

In equation (2), $x$ is the weight gained from the beginning of any cycle up to any age, $t$, of that cycle; while $A$ is the total growth made in that cycle which is equivalent to the limiting growth substance present at the beginning of the cycle. If the cycle under consideration is preceded by one or more other cycles, then the growth of the preceding cycles must be subtracted from the weight of the animal at the age $t$ in order to obtain $x$. Let $w$ be the weight gained up to the beginning of the cycle under consideration and $x_1$ the weight of the animal at the age $t$; then equation (2) becomes

$$\log \frac{x_1 - w}{A - (x_1 - w)} = K(t - t_1) \qquad (3)$$

The simplest method of applying equation (2) or (3) is first to plot the experimental data on growth in terms of monthly gains as was done in Figs. 2 and 3. The highest point of the velocity curve corresponds by definition to the age $t_1$. Having thus evaluated $t_1$, a reasonable beginning or end of the cycle is chosen by inspection; the growth made between the ages $t_1$ and the beginning or end of the cycle is the value of $\frac{1}{2}A$, since $t_1$ is in the center of the growth cycle. If the values of $t_1$ and $\frac{1}{2}A$ are properly chosen, the values of $K$ for different values of $x$ should be nearly constant. If the value of $K$ deviates systematically then a slightly smaller or slightly larger value of $\frac{1}{2}A$ is chosen until the systematic deviations disappear; the

[7] *Cf.* Lewis, G. N., *Z. physikal. Chem.*, 1905, lii, 310; *Dept. Interior, Bureau Gov. Lab., Chem. Lab., No. 30*, 1905.

value of $A$ can be estimated still more easily by changing equation (2) $\log \frac{x}{A-x} = Kt$ to $y = Kt$ when $y$ stands for $\log \frac{x}{A-x}$. Since $y = Kt$ represents a straight line therefore $\log \frac{x}{A-x}$, if plotted against $t$, should also give a straight line for the proper value of $A$. A few values of $A$ are chosen and a few values of $\log \frac{x}{A-x}$ are plotted conveniently with the aid of semilogarithmic paper or still better by the aid of Robertson's tables.[8] The value of $A$ giving the straightest line is the one chosen for computation. The last graphic method of verifying the correctness of the estimated value of $A$ was called to our attention by Dr. E. R. Hedrick of this University, and it has proved very satisfactory in this work.

The first extrauterine cycle, $A$, should, in accordance with the tricyclic theory of growth, be preceded by another cycle *in utero*; that is, $w$ in equation (3) should have some value other than zero. Since, however, we have no data on intrauterine growth of the dairy cow and since the neglect of the relatively small gain in the intrauterine cycle cannot affect the results very seriously, we shall for the present assume $w = 0$ and the weight gained up to $t_1$ is $\frac{1}{2}A$. Assuming the value of $t_1$ for the Holstein cow to be 4.5 months when the animal weighs 275 pounds (see Fig. 3), we get the value for $A$, $275 \times 2 = 550$ pounds, and equation (2) for the Holstein cow takes the form

$$\log \frac{x}{550-x} = 0.158\,(t - 4.5)$$

which should enable computation of comparable values of the animal to at least 9 months ($2 \times 4.5$) of age which in fact is the case as shown in Table II.

After 9 months of age the observed values are greater for the probable reasons previously explained.

By the same method the equation for the Jersey cow is found to be

$$\log \frac{x}{444-x} = 0.169\,(t - 5)$$

[8] Robertson, T. B., *Univ. California Pub. Physiol.*, 1915, vi, 211.

This equation gives an equally satisfactory agreement between the calculated and observed average values for the Jersey cow as seen in Table III.

TABLE II.

| Age ($t$). | Weight observed ($x$). | Weight calculated ($x$). |
|---|---|---|
| *mos.* | *lbs.* | *lbs* |
| 0 | 90 | 90 |
| 1 | 121 | 120 |
| 2 | 157 | 157 |
| 3 | 200 | 202 |
| 4 | 249 | 250 |
| 5 | 302 | 301 |
| 6 | 349 | 348 |
| 7 | 389 | 393 |
| 8 | 425 | 430 |
| 9 | 466 | 461 |
| 10 | 501 | 485 |
| 11 | 529 | 503 |

TABLE III.

| Age ($t$). | Weight observed ($x$). | Weight calculated ($x$). |
|---|---|---|
| *mos.* | *lbs.* | *lbs.* |
| 0 | 55 | 55 |
| 1 | 76 | 76 |
| 2 | 105 | 105 |
| 3 | 140 | 140 |
| 4 | 174 | 180 |
| 5 | 222 | 222 |
| 6 | 260 | 265 |
| 7 | 302 | 305 |
| 8 | 340 | 340 |
| 9 | 376 | 367 |
| 10 | 407 | 389 |

The velocity curves of the last cycle, B (in Figs. 2 and 3) are very irregular, and no significance could be attached to the fit of equation (3) for the data of that period. The possible causes of their irregularity have been indicated. It might, however, be of interest to apply equation (3) to the data in order to illustrate the method of procedure. If the growth of the Jersey cow is taken as example it

would seem reasonable from inspection of Fig. 2 to assume the value of $t_1$, the maximum, as 20.5 months of age when the animal weighs 635 pounds—the average weight of the Jersey cow at maturity having been found by Eckles[5] to be 902 pounds. $A$ is therefore $2 \times 267 = 534$ pounds, and $w = 902 - 534 = 368$ pounds. Equation (3) after evaluation of $K t_1$ becomes

$$\log \frac{x_1 - 368}{534 - (x_1 - 368)} = 0.0765\,(t - 20.5)$$

Calculating $x$ as before, we get Table IV.

TABLE IV.

| Age ($t$). | Weight observed ($x$). | Weight calculated ($x$). |
|---|---|---|
| *mos.* | *lbs.* | *lbs.* |
| 15 | 520 | 513 |
| 16 | 533 | 533 |
| 17 | 553 | 553 |
| 18 | 572 | 575 |
| 19 | 598 | 598 |
| 20 | 621 | 621 |
| 21 | 649 | 645 |
| 22 | 668 | 669 |
| 23 | 689 | 690 |
| 24 | 716 | 713 |
| 25 | 737 | 734 |
| 26 | 758 | 754 |
| 27 | 770 | 771 |
| 28 | 784 | 788 |
| 29 | 804 | 803 |

If all the assumptions are correct, and if pregnancy under the conditions at which the animals were kept did not interfere with their growth, then the observed weights before calving, at 29 months of age, should be greater by at least 55 pounds, the weight of the calf at birth, than the calculated weights. The effect of pregnancy on growth, however, is not well known, the evidence being somewhat contradictory,[9] and no conclusions can therefore be drawn from this rather too close agreement.

[9] *Cf.* Marshall, F. H. A., The physiology of reproduction, London, 1910. Minot, C. S., *J. Physiol.*, 1891, xii, 97. Watson, J. B., *J. Comp. Neurol. and*

Table V shows the calculated and observed values during lactation. The large deviation of the calculated values from the observed values is in agreement with the established fact that lactation greatly retards growth.[9]

TABLE V.

| Age ($t$). | Weight observed ($x$). | Weight calculated. |
|---|---|---|
| *mos.* | *lbs.* | *lbs.* |
| 29 | 764 | 803 |
| 35 | 779 | 863 |
| 42 | 827 | 890 |
| 48 | 854 | 898 |
| 55 | 872 | 900 |
| 68 | 887 | 902 |
| 80 | 902 | |

## SUMMARY.

The growth period of the Jersey and Holstein cows is made up of at least three cycles, two extrauterine cycles with maxima at about 5 and 20 months of age, and one intrauterine cycle, the maximum of which has not yet been determined. The equation of an autocatalytic monomolecular reaction was found to give very good results when applied to the cycle having its maximum at about 5 months of age. The values obtained from this equation when applied to the cycle having the maximum at about 20 months of age were higher than the observed values probably due to the retarding effect of pregnancy and lactation on growth.

We are indebted to Dr. Walter R. Bloor and Dr. Carl L. A. Schmidt, Department of Biochemistry, University of California, for valuable suggestions.

*Psychol.*, 1905, xxv, 514. Eckles, C. H., *Univ. Missouri Agric. Exp. Station, Bull. 135*, 1915. Eckles, C. H., and Swett, W. W., *Univ. Missouri, Agric. Exp. Station Research Bull. 31*, 1918. Regan, W. M., The effect of lactation on growth, Thesis, Columbia, 1914. Griswold, D. J., A study of the effects of the periods of gestation and lactation upon growth and composition of swine, Thesis, Columbia, 1915.

# CASEIN VISCOSITY STUDIES.*

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## INTRODUCTION.

Robertson[1] has shown that when casein is dissolved in dilute acids or alkalies (HCl or KOH), the viscosity of the solution increases with the increase in the concentration of the solvent. A similar set of results were obtained by Sackur.[2] That a maximum viscosity occurs in solutions of casein in alkalies has been pointed out by the writer upon another occasion.[3] Detailed publication of these results has been delayed because it was subsequently found that borax caused a displacement of this maximum viscosity[4] and it was very desirable to repeat with care the determination of the viscosity curves in order to be sure that no error in observation had been committed in the earlier experiments.

The viscosity-pH curves of casein in alkaline solutions only are considered in this paper. Viscosity-pH curves of pure casein in acids have been observed by the writer, but they are lacking in the practical significances manifested by the alkaline solutions. It may not be remarkable that all the commercial casein glues examined yielded mixtures, the pH values of which showed that the maximum viscosity had been widely overstepped, and that the viscosities of the mixtures lay on the alkaline flat part of the curve. The practical importance of knowing at what pH casein solutions show their maxi-

* Published by permission of the Secretary of Agriculture.

[1] Robertson, T. B., The physical chemistry of the proteins, Washington, 1918, 320–328.

[2] Sackur, O., *Z. physikal. Chem.*, 1902, xli, 672.

[3] Zoller, H. F., *Science*, 1919, l, 49.

[4] Zoller, H. F., *J. Ind. and Eng. Chem.*, 1920, xii, 1171.

mum of internal friction outcrops in all industries where casein is used in the form of its solutions; *e.g.*, paper coating, sizings, paints, adhesives, nutritive preparations, dentrifrices, etc.

Two features are brought out in the viscosity curves which deserve separate treatment. On the one hand, as above mentioned, when casein is dissolved in sodium borate the maximum viscosity is attained at about pH 8.1 instead of pH 9.2 as is true for NaOH, KOH, and LiOH. Therefore a study of the anion effect is reported in Section II of these studies. Upon the other hand we observe a characteristic decline in the viscosity following the maximum and this decline is followed by a broad flattening of the curve. This effect has been correlated with the alkaline hydrolysis of the casein, whence numerous cleaved bodies are thrown into solution, such as ammonia, sulfides, phosphates, albumoses, etc. With the production of ammonia there is a flattening of the curve which has further significance when it is pointed out that solutions of casein in ammonia show a maximum viscosity, but this maximum, once attained at pH 9.2, continues without the characteristic decline. Thus, it will be appreciated that solutions of casein in ammonia generally maintain a higher viscosity. In Section III, therefore, the effect of alkaline hydrolysis upon the viscosity of casein will be dealt with separately.

Section IV will treat very briefly of the influence of high temperatures upon the viscousness of casein solutions, inasmuch as the author has treated of this factor somewhat more in detail in connection with the revision of the borax solubility test for caseins.[4]

### *I. Relation Between pH and Viscosity of Solutions of Casein in Alkalies.*

Since the earlier investigators in dealing with casein solutions made no attempt to study the effect of hydrogen ion concentration upon their viscosity it was found expedient to redetermine the viscosity curves of casein in alkalies using the hydrogen electrode as a check upon the reaction of the solutions. Chick and Martin[5] measured the effect of concentration of casein upon the viscosity but did not indicate whether or not the reaction of the solutions was held at constant pH. Consequently the writer has repeated these measurements,

[5] Chick, H., and Martin, C. J., *Kolloid. Z.*, 1912, xi, 102.

being certain that the reaction of the casein solution was the same in all concentrations. pH 9.0 was chosen for these measurements because at this point we are in the region of maximum viscosity of casein in the ordinary alkalies.

*Preparation of Solutions.—Alkalies.*—Sodium, potassium, and lithium hydroxides were freed as far as possible from their carbonates by preparing concentrated solutions and allowing the carbonates to crystallize, then filtering or pouring the clear solutions from the precipitate. Molar solutions were then prepared of the respective compounds; M/1 solution of freshly distilled $NH_3$ was also prepared.

*Casein.*—Casein was obtained by purifying a quantity of imported casein according to the method of Hammarsten as modified by Robertson.[1] This was designated by the symbol *R*. A casein, the history of which was completely known, so as to overcome the question of heated casein, was prepared by precipitating the casein from fresh, unpasteurized skim milk with dilute HCl according to the grain curd method.[6] The casein was then dissolved in ammonia and reprecipitated twice in order to free it from salts and other impurities. It was then dried with concentrated alcohol without the use of heat. This casein was designated as *G* in the following studies.

In the preparation of all casein solutions for the different studies the casein was carefully weighed into a dry flask and 50 cc. of distilled water, which had been freshly boiled and cooled by drawing $CO_2$-free air through it while immersed in running cold water, were added with thorough shaking to prevent the finely divided casein from forming lumps. After soaking in the water for 30 minutes the requisite quantity of alkali (M/1) was added from a burette. The mixtures were set in a water bath controlled at 30°C. and when all the casein had dissolved (2 hours were allowed) they were made up to final volume with the distilled water described above.

*Measurement of Viscosity.*—Although many of the measurements of viscosity of casein solutions have been conducted in the past with the usual type of capillary viscosimeter the present work was done almost entirely with a rotating disc instrument. The convenience of this latter type of instrument for the heavy casein solu-

[6] Clark, W. M., Zoller, H. F., et al., *J. Ind. and Eng. Chem.*, 1920, xii, 1163.

tions is at once appreciated and for comparative measurements of such protein solutions is probably more nearly representative of their true internal friction because the measurements can be made quickly and with little molestation of their physical structure.[7]

The writer chose the MacMichael viscosimeter which embraces the rotating disc because of its availability to those interested in checking the viscosity values of casein solutions. The double brass bowl furnished with the instrument was replaced by one designed especially for these investigations. The essential difference was the provision for accurately controlling the temperature of the water bath in the outer chamber so that the viscosity measurements could be made at known temperatures. Fig. 1 is a cross-sectional diagram of the bowl used. A represents the removable inner bowl of brass for holding the solution under measurement; B, the outer bowl which is made to fit on the turntable of the instrument; C, the brass supports for the inner bowl; D, a small spiral of nichrome wire wound around a silica tube for the heating unit, and is inclosed in a small tube of pyrex glass; E, a small mercury thermoregulator. D and E are suspended in the water bath F and held rigid; the rotation of the bowl by the driving mechanism produces the stirring effect. D and E are further operated through a relay with carbon lamps in series in the circuits. G is the suspended disc furnished with the instrument. (For further comments on the MacMichael viscosimeter see Herschel.[8])

The values given for the viscosity of the various casein solutions are those obtained by subtracting the viscosity of the solvent at the temperatures measured from the total viscosity of the solution expressed in the divisions marked on the dial of the instrument. They are not absolute values, therefore, but only relative; the same relation would doubtless hold for any type of viscosimeter. The viscosity was measured at 25°C.

*Determination of pH.*—In the first instances of the work the reaction of the casein solutions was gauged by the use of the sulfonephthalein indicators similar to the method since described by Haas.[9] Five drops of the casein solution were placed on the inner surface of

[7] Garret, H., *Phil. Mag.*, 1903, vi, 374.
[8] Herschel, W. H., *J. Ind. and Eng. Chem.*, 1920, xii, 282.
[9] Haas, A. R. C., *J. Biol. Chem.*, 1919, xxxviii, 49.

a small porcelain crucible lid and one drop of a water solution of the indicator was added (0.04 per cent indicator solution). This was compared to the same quantity of indicator in a mixture of four drops of buffer standards (Clark and Lubs standards) plus one drop of 1 per cent starch paste, on a similar background.

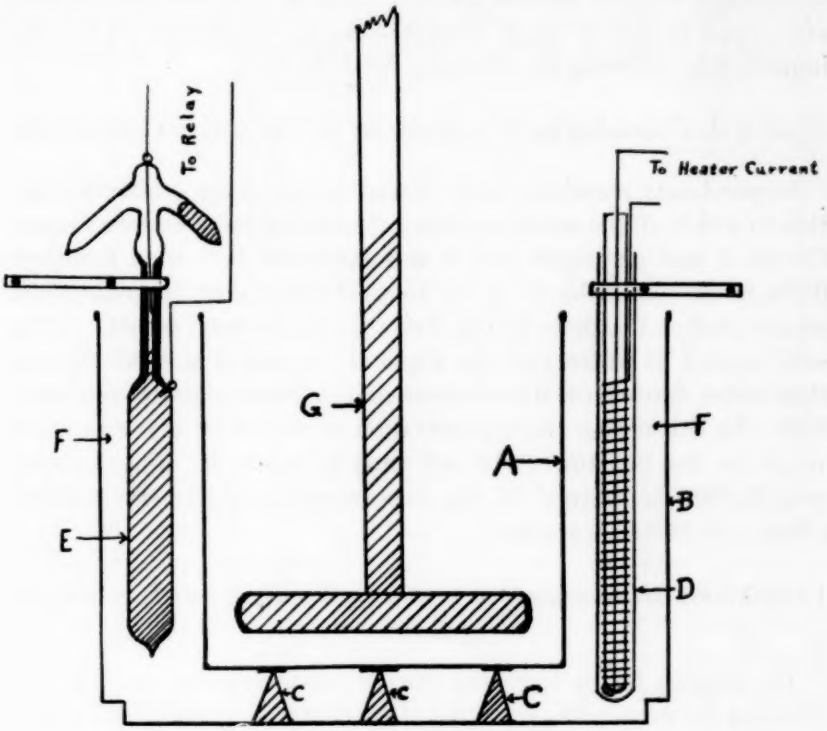


FIG. 1. Viscosimeter bowl.

It was later found that the values given by the above method were incorrect and the error was not constant but varied with the indicator and with the region of the pH scale. The hydrogen electrode was used, it consisted of the rocking electrode type described by Clark,[10] using saturated KCl junction and saturated calomel electrode. The platinum electrodes were first plated with gold and then with

[10] Clark, W. M., The determination of hydrogen ions, Baltimore, 1920.

a smooth coat of iridium. A well plated electrode lasted during one complete run of a single series of casein solutions titrating from the acid end of the series toward the alkaline end, usually about eight consecutive determinations.

The hydrogen ion concentrations of the various solutions were determined without dilution at 30°C. They were also determined after equal time intervals of contact of casein and solvent ($2\frac{1}{2}$ hours), immediately following the viscosity determination.

*Effect of the Concentration of Caseinate on the Viscosity at Constant pH.*

As previously mentioned pH 9.0 was chosen as the arbitrary reaction to which all the solutions were adjusted for these measurements. Casein *R* was employed and it was measured into wide mouthed flasks in the quantities necessary to yield the various concentrations represented on the curve in Fig. 2 as the experimental points. They were soaked in water, and the required amount of M/1 NaOH was then added to reach pH 9.0 (electrometric) after adjusting to final volume. In calculating the concentration of caseinate, allowance was made for the moisture (2.10 per cent) in casein *R*. Fig. 2 shows graphically the nature of the concentration effect. It confirms Chick and Martin's results.[5]

*Viscosity-pH Measurements of Casein in NaOH, $NH_4OH$, KOH, and LiOH.*

The interest in the foregoing viscosity-concentration curve lies in choosing the proper concentration of caseinate solutions for the extensive viscosity measurements. By reference to Fig. 2 we find that a 9 to 10 per cent caseinate solution is in the region of maximum sensibility to differences in reaction and to other factors such as salts, or ion effects. It was decided, therefore, to dissolve 10 gm. of Casein *R* or *G* in 100 cc. of solvent solution in every case. As the alkali was varied in each mixture the water was varied correspondingly so that the total volumes of the two were always 100 cc. This gave solutions containing approximately 9 per cent of caseinate.

For brevity the tables of each individual set of experiments are omitted and the data as far as possible are presented in Fig. 3. Only

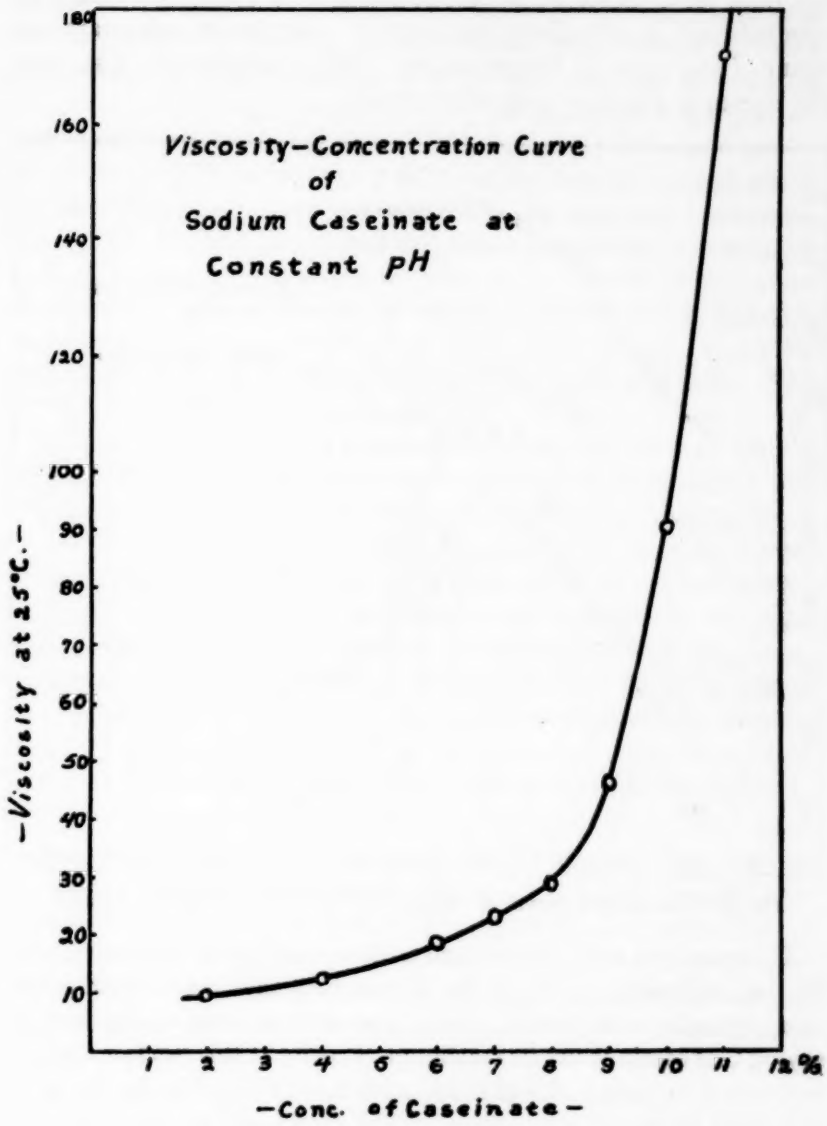


FIG. 2. Viscosity-concentration curve.

the data for *G* dissolved in NaOH are included, since *G* seemed to conduct itself in all respects similar to *R*. All the pH values are the result of electrometric determination. Fig. 3 contains the viscosity-pH curves of the four hydroxides studied.

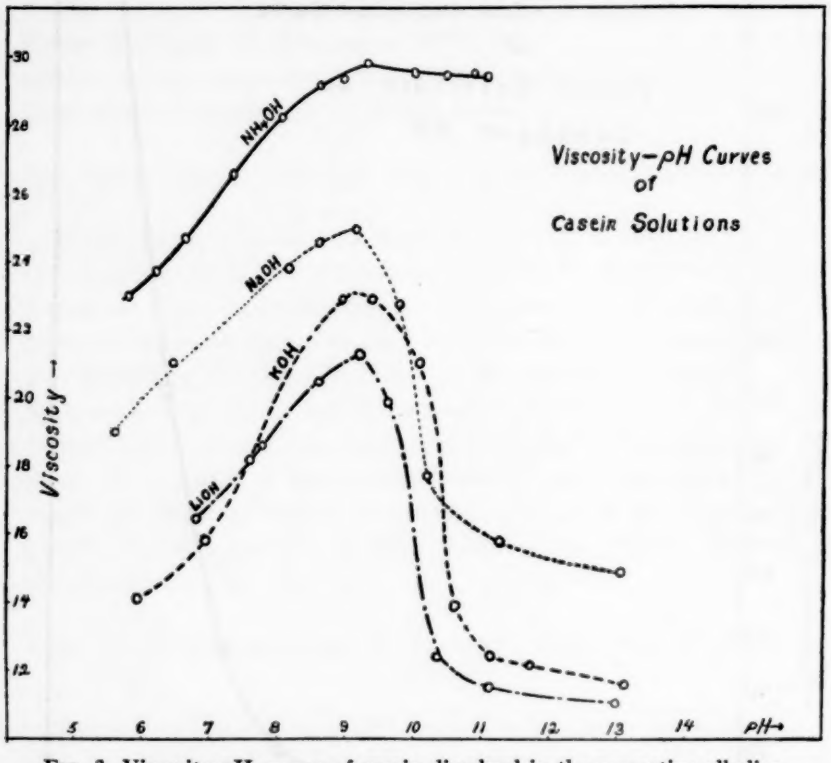


FIG. 3. Viscosity-pH curves of casein dissolved in the respective alkalies.

There are two noticeable characteristics among the various curves. In the proximity of pH 9.0 the different alkalies reach their maximum viscosity with casein. This is so uniform that we are safe in saying that casein shows its maximum viscosity at this reaction except, as the data in Section II will show, when it is dissolved in the presence of certain anions such as borates. The magnitude of this maximum viscosity varies somewhat with the alkali, being greatest with $NH_4OH$ and least with LiOH, though the difference is apparently of little immediate importance.

The viscosity drops precipitously following the maximum with each alkali except $NH_4OH$. In this case there is a slight fall but it continues to remain high even in great concentrations of ammonia (50 cc. of M/1 to 10 gm. of casein). At pH 10.5 with NaOH, KOH, and LiOH there is an evolution of $NH_3$ from the solutions, shown by white fumes with concentrated HCl and by turning methyl red to yellow (methyl red paper was held above the solution). Following pH 10.5 the viscosity is constant and about parallel to the ammonium hydroxide curve. This serves to show that in these concentrations of alkali the casein is furnishing $NH_3$ to the solutions so that they simulate the influence of the pure ammonium curves. Hence the flattening could be attributed to the accumulation of $NH_3$. This will be dealt with further in Section III.

Robertson calls attention to the relation between the ionization of protein solutions and their viscosity.[1] The viscosity increases with the electrolytic dissociation of the proteins; of course as ionization is conceived to be zero (or in electrochemical equilibrium) at the isoelectric point, we find simultaneously with the isoelectric condition a minimum viscosity. Hence Robertson points out[1] that at the point of maximum base-binding capacity, or saturation with base, there should be a maximum viscosity. If we note the quantity of NaOH that is necessary to cause the casein to attain its maximum viscosity at pH 9.2 we find that for each gram of moisture-free casein 0.98 cc. of normal NaOH was required (average of eight determinations). At this point 1 gm. of casein had combined with $98 \times 10^{-5}$ gram equivalents of alkali. This value is slightly greater than one-half the value obtained by Robertson ($180 \times 10^{-5}$) using the gas chain method.[11] The writer employed very concentrated solutions (8 to 10 per cent) of casein, while Robertson worked with very dilute solutions and this may be responsible for the difference in observations. In two instances, the writer allowed the concentrated casein solutions

[11] Robertson, T. B., *J. Physical Chem.*, 1910, xiv, 528.

The high concentrations of casein used by the writer have given values for the pH of the maximum viscosity, considerably different from those observed by Loeb in working with dilute solutions. (Loeb, J., *J. Gen. Physiol.*, 1921, iii, 357.) The interval between the preparation of the casein solution and the determinations of the pH seemed ample for the equilibrium.

to stand for 30 hours, but the relative values remained the same; that is, the maximum viscosity occurred when the casein had combined with $90-100 \times 10^{-5}$ gram equivalents of base. When $180 \times 10^{-5}$ gram equivalents of alkali were added to 1 gm. of casein in the writer's studies, the viscosity had fallen to a minimum, or on the flat part of the curve, and the pH was greater than 10.5. This would indicate that hydrolysis is taking place at this combining capacity, or at least it is in the first stages.

*II. Anion Influence on the Viscosity of Casein Solutions.*

The anions studied in this connection were those resulting from the ionization of the following salts: $Na_3PO_4$, NaF, $Na_2SO_3$, $Na_2B_4O_7$, $Na_2CO_3$, $Na_3AsO_4$, and $Na_2SiO_3$. Casein is readily soluble in solutions of the above salts. It is also readily soluble in solutions of the neutralized acids (neutral sodium salts), such as citric, oxalic, tartaric, etc., but these viscosity data are not presented.

The method used in preparing the casein solutions was essentially the same as that for the pure alkalies described in Section I. The casein was first soaked in 50 cc. of water and the solvent then measured into each quantity; they were then made up to volume. After the regular time interval ($2\frac{1}{2}$ hours), the viscosity and hydrogen ion concentration were measured exactly as in Section I. Fig. 4 shows the viscosity curves plotted from the experimental data.

The curve for sodium carbonate is necessarily similar to the NaOH curve. Naturally, if the curves represented the data for viscosity plotted against concentration of salt solution instead of pH we would observe wide variations in the gradient of the various curves corresponding to the differences in the dissociation of the solvents. Since the degree of internal friction varies more pronouncedly with the pH than with the concentration of molecules of the solvent, we are correct in representing the values as given in Fig. 4. It was impossible to use the hydrogen electrode in the sodium sulfite solutions for the determination of pH, because of fluctuating potentials probably resulting from sulfur dioxide which permeates the solutions. The dissociation constant of sulfurous acid is less than that of casein and this accounts for the formation of $SO_2$ in the solutions. The pH was determined roughly by the spot test with sulfonephthalein indicators.

The curve for sulfite is not presented because of the inaccurate pH data. But a maximum viscosity was obtained, using the same time interval as employed in the other data, in a region corresponding to a pH of 6.4 to 6.6. What this may mean is difficult to interpret with the meager data at hand. No attempt was made to remove $SO_2$ from the solutions before taking measurements.

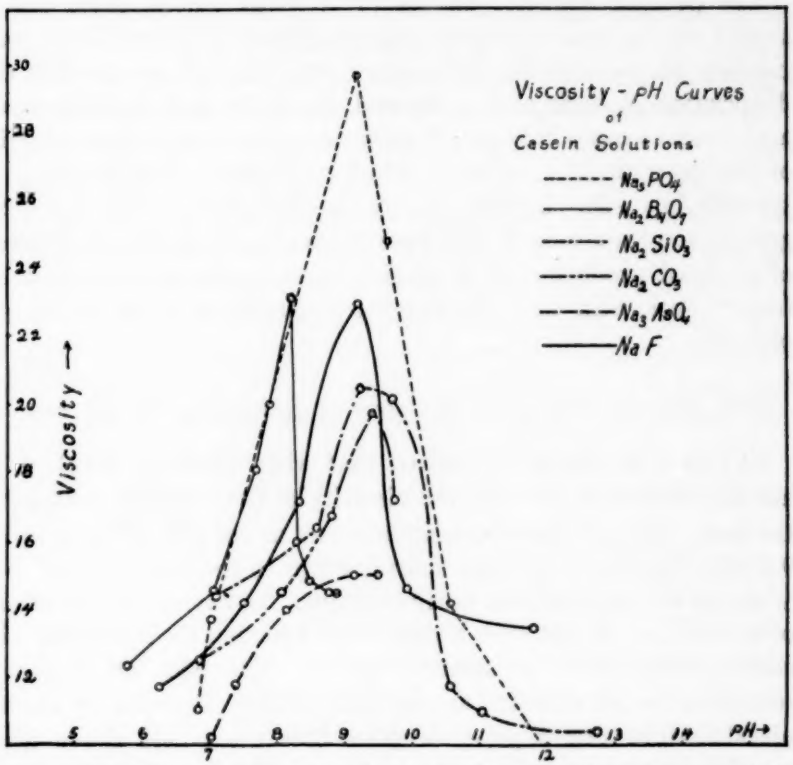


FIG. 4. Viscosity-pH curves of casein dissolved in the various solvents. Anion effect on the viscosity maximum.

With sodium arsenate a precipitation was observed at the point of maximum viscosity; the precipitate increasing in quantity with the increasing pH. The hydrogen electrode potentials were very steady. On the other hand sodium silicate solutions of casein gave unsteady potentials, although they were somewhat more steady than with sulfite. The pH values for sodium silicates as given are correct within 0.1 pH.

Many of these salts are employed in casein water-resisting glues to combine with the excess of calcium oxide used as solvent for the casein. Certain ones will effect the setting time of the glues, thereby regulating the smoothness of the product. The above data on viscosity influence of these salts should indicate the choice of correct solvents for particular purposes.

The peculiar behavior and place of borax among these curves should not be passed without remarking that the phenomenon may possibly be traced to the influence of polyhydroxyl groups upon the dissociation of boric acid. Glycerol, mannitol, and various sugars are known to cause increased dissociation of boric acid which permits of the determination of boric acid by titration. The presence of tyrosine in casein suggests that casein may contain free hydroxyl groups, and these have indeed been demonstrated by the preparation of acylated products. It is possible that casein will act similarly toward boric acid thus effecting the displacement of the maximum viscosity.

*III. Alkaline Hydrolysis of Casein as Influencing Its Viscosity.*

Section I of this series showed that when casein is dissolved in alkalies other than $NH_4OH$ the viscosity of the solutions containing the same concentration of casein increases as the pH increases up to 9.2 after which the viscosity sharply drops to the region of pH 10.5 after the viscosity remains fairly constant. In case of $NH_3$ solutions of casein there is not such a drop after reaching the maximum viscosity (temperature of solutions 25°C). The odor of all casein solutions, the pH of which are pH 10.0 or more, is distinctly ammoniacal. Moist litmus paper becomes blue when held above these solutions, while strong HCl evolves dense white fumes when brought in their vicinity.

Dakin and Dudley[12] have prepared what they term racemized casein by treating casein with 2 per cent NaOH for several hours, or until the specific rotation has decreased to zero. They observe that ammonia is evolved, that the racemized casein contains a low content of phosphorus, and describes the process of decrease in rotation

[12] Dakin, H. D., and Dudley, H. W., *J. Biol. Chem.*, 1913, xv, 263.

as one of racemization or a keto-enolic tautomerism. Whatever explanation might serve to describe the change taking place in the molecule to effect the optical properties, there is a far greater fundamental change taking place in the casein molecule in the presence of these concentrations of alkalies which has received little attention. Maynard[13] has shown that NaOH of 2 per cent concentration also cleaves loosely combined sulfur from the casein molecule, a fact apparently noted by Dakin and Dudley.[12]

The writer has been able to prepare an acid-precipitable product, from the action of 2 per cent NaOH upon casein, that contains no sulfur, no nitrogen in the free amino form, and only a trace of phosphorus. The action of the alkali upon the casein proceeded for 6 days at 30°C. The gelatinous solution was diluted with water and treated with dilute acetic acid. The curd or precipitate was collected and thoroughly washed with water containing a trace of acetic acid. It was then thoroughly washed with distilled water and dried to constant weight, and analyzed for amino nitrogen, sulfur, and phosphorus. The action of alkali upon the casein has been more severe than a racemization or enolization, and it is quite likely that the loss of amino nitrogen, sulfur, and phosphorus from the casein molecule is partially responsible for the decline in rotation observed by Dakin and his coworkers.

In applying this knowledge to the explanation of the flattening of the viscosity curves in the alkaline zone the writer has found that the cleavage of sulfur and phosphorus is simultaneous with the cleavage of amino nitrogen. They both commence at nearly the same intensity of hydroxyl ion concentration, or, reciprocally, at about the same pH (10.0 to 10.5). The hydrolysis also commences as soon as the casein is dissolved in solvent at this pH. Within 6 hours nearly ½ the phosphorus and sulfur had been cleaved from the casein at pH 11.1 (9 per cent casein solution). When a few drops of $CuSO_4$ solution are added to the flasks containing solutions of casein for viscosity measurements, it is observed that the characteristic biuret color develops within a few minutes in those solutions which are found to evolve ammonia, and the depth of violet color increases with time and

[13] Maynard, L. A., *J. Physical Chem.*, 1919, xxiii, 145.

with increasing pH. All casein solutions at pH 10.0 to 10.5 and higher pH responded to the copper reaction; if dilute acid is added to the casein solutions, which contain copper, and give the violet color, a dark color is formed owing to the formation of peptized copper sulfide.

The protein body which is precipitated from the alkaline hydrolysate of casein by the addition of dilute acids is, as described by both Dakin and Maynard, very gelatinous. It forms a clear transparent gel of high viscosity. The formation of this hydrolytic product from casein in the presence of the liberated $NH_3$ is believed to be responsible for the flattening of the viscosity curve at pH greater than 10.0.

The quantity of nitrogen split off from casein by the alkaline hydrolysis was found to be nearly $\frac{1}{2}$ the lysine nitrogen of the original casein and designated as free amino nitrogen by Van Slyke and Birchard.[14]

It can be stated with a sense of security that in solutions originally containing casein of which the pH are greater than 10.0, we are no longer dealing with casein but with protein-cleaved products. The flattening of the viscosity curve is coincident with the alkaline hydrolysis of the casein.

Furthermore the glues and adhesives formed from casein are ultimately a mixture of these cleaved products. It seems necessary now to determine the relative adhesive power of pure caseinate and the protein body which constitutes the larger bulk of the casein alkaline hydrolysate.

### *IV. Temperature as Effecting the Viscosity of Casein Solutions.*

If we heat solutions of casein in alkalies (NaOH) to the boiling point and allow the solutions to cool spontaneously the resulting viscosities as measured at 25°C. are very little different from the viscosities of the unheated solutions at equivalent pH. Upon the other hand if the casein is precipitated from its solutions, after they have been heated, by acid and is then dried the resulting casein again dissolves in alkalies to furnish solutions of much higher viscosity.

[14] Van Slyke, D. D., and Birchard, F. J., *J. Biol. Chem.*, 1913, xvi, 539.

Upon another occasion the writer has shown the effect of the heat treatment of milk upon the optimum temperature for acid precipitation of casein[15] and at the same time presented some data to show that this denaturation of the casein affected its hydrophyllic properties.[6,15] Again the conduct of casein, heated and unheated, toward borax solutions in the borax solubility test[4] emphasized that casein which had been subjected to high temperatures imbibes more water in solution and thereby produces greater internal friction or viscosity; in many instances they gel where normal casein solutions flow readily. The writer has further shown the formation of $\beta$ casein in highly heated casein solutions.[16]

To show the effect of heat treatment of casein upon its viscosity in NaOH several experiments were conducted, two of which are described. Fresh skim milk was divided into two portions, *A* and *B*. The casein was precipitated from *A* according to the normal grain curd method[6] without heating the milk externally above 34°C. *B* was pasteurized at 65°C. for 45 minutes and the casein was precipitated from it with the modified grain curd method described by the writer.[14] The two moist caseins were dried under the same conditions in an air current at 40°C. The viscosities of the resulting caseins $A_e$ and $B_e$ were measured at 25°C. in the MacMichael apparatus as modified. Casein $B_e$ contained 0.45 per cent more ash than $A_e$.

EXPERIMENT I.

| Casein. | Viscosity. | pH | Concentration of casein. |
|---|---|---|---|
| | | | *per cent* |
| $A_e$ | 39.2 | 9.15 | 8.8 |
| $B_e$ | 49.3 | 9.12 | 8.7 |

The other experiment mentioned above consisted in pasteurizing a 6.0 per cent solution of Casein *R* dissolved in NaOH at 80°C. for 30 minutes. The solution was cooled thoroughly and its viscosity at 25°C. was compared to the viscosity of a similar solution that had not been pasteurized.

[15] Zoller, H. F., *J. Ind. and Eng. Chem.*, 1921, in press.
[16] Zoller, H. F., *Chem. Abst.*, 1921, xv, 401–02.

EXPERIMENT II.

| Casein. | Viscosity. | pH |
|---|---|---|
| Pasteurized solution | 25.4 | 6.43 |
| Unpasteurized solution | 27.6 | 7.16 |

These two experiments are typical of what we can expect of the effect of heat upon casein as affecting its viscosity. The results at

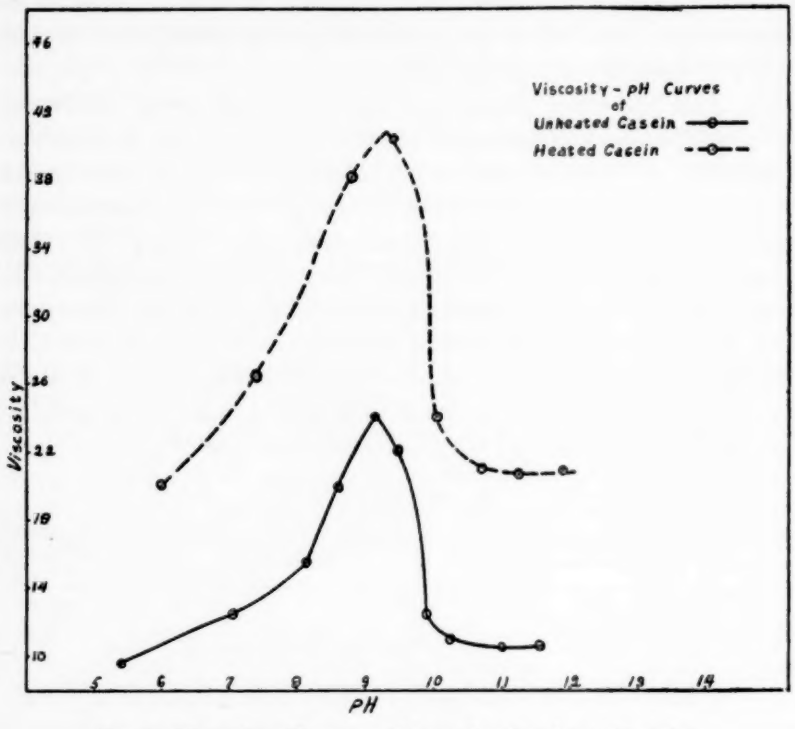


FIG. 5. Viscosity-pH curves of heated and unheated caseins.

first seem to be contradictory. But in the light of viscosity data obtained with milk the results of Experiment 2 show that the decreased viscosity by pasteurizing can be entirely accounted for through the decreased pH of the solutions, provided that we ignore the effect of heat on the coaguable proteins and casein. In milk, pasteurization generally causes a drop in pH of 0.10 to 0.20 and a drop in viscosity

with the MacMichael instrument from 71.0 before pasteurizing to 69.0 after pasteurization. When the casein is separated from the milk serum salts and dried, its hydrophyllic properties are changed and it can then imbibe more water. Inasmuch as borate solutions buffer in the region of maximum viscosity of casein solutions it is peculiarly well suited to the differentiation of heated and unheated caseins, from the standpoint of their power of imbibition.

Fig. 5 shows the viscosity-pH curves of two caseins; one of a casein prepared without heat treatment, the other of some of the same casein precipitated from NaOH solution (pH 7.2) after the solution had been boiled for 20 minutes; the casein was dried with alcohol. The viscosity of the heated casein is nearly twice that of the unheated. The conditions of measurements were the same and the solvent was M/1 NaOH.

## SUMMARY.

1. Viscosity and pH curves of casein dissolved in NaOH, KOH, LiOH, and $NH_4OH$ are shown and it is found that a maximum viscosity occurs at about the same pH point with each alkali; *i.e.*, 9.1 to 9.25. The magnitude of the viscosity is largest in ammonia solutions.

2. The maximum viscosity occurs in 8 to 10 per cent solutions of casein in alkalies when about $98 \times 10^{-5}$ gram equivalents of base are combined with 1 gram of casein.

3. A maximum viscosity occurs in the same region (pH 9.1 to 9.25) when casein is dissolved in $Na_2CO_3$, $Na_3AsO_4$, $Na_2SO_3$, NaF, and $Na_2PO_3$.

4. The maximum viscosity obtained with borax solutions of casein occurs at 8.15 to 8.2 pH. It is suggested that casein acts like mannitol, glycerol, etc., in increasing the dissociation of boric acid.

5. The flattening of the viscosity curves of casein solutions, following the decline from maximum, is shown to be due to alkaline hydrolysis whence casein no longer exists as such but is cleaved into a major protein containing no phosphorus or sulfur and less nitrogen. This cleavage commences at pH 10.0 to 10.5.

6. When casein is prepared from solutions that have been subjected to high temperatures (60°C. and above) or has otherwise been heated during its preparation, it yields solutions in alkalies of high viscosity.

# THE SIGNIFICANCE OF LATENCY TIME IN ENZYME DETERMINATION.

By LOURENS G. M. BAAS-BECKING.

*(From the Department of Botany, Stanford University, Pacific Grove.)*

(Received for publication, January 18, 1921.)

Bredig and von Berneck,[1] in their work on inorganic ferments, determined the catalytic activity of platinum and manganese salts on hydrogen peroxide by means of titration with potassium permanganate. In many of their experiments they found, however, that the reaction velocity increased during the reaction. Bredig and Marck[2] attempt to explain this fact by a gradual activation of the catalyzer by the substrate. Denham[3] believes that the increase in reaction velocity is due to absorption at the surface layer. The results of Waentig and Steche[4] on blood catalase seem to indicate that the increase in reaction velocity is not a constant feature of the catalase; they found a constant, or even a decreasing, reaction velocity. Senter,[5] however, obtained opposite results with the same enzyme; there were cases in which the velocity increased. It seems to me that the controversy between different authors can, at least partially, be solved by the following considerations.

As I was able to demonstrate, the reaction does not start immediately after mixing the hydrogen peroxide with the enzyme solution. For this experiment I used an autographic registration of catalase action in a manometer. A certain latency period shows itself before the reaction starts. Probably an adsorption compound is formed during this latency period, which on decomposition liberates the oxygen. In one case, I obtained a latency time of 98 seconds while

[1] Bredig, G., and von Berneck, R. M., *Z. physikal. Chem.*, 1899, xxxi, 258.

[2] Bredig, G., and Marck, A., Gedenkboek aangeboden aan J. M. Van Bemmelen, 1910, 342.

[3] Denham, G. H., *Z. physikal. Chem.*, 1910, lxxii, 686.

[4] Waentig, P., and Steche, O., *Z. physiol. Chem.*, 1911, lxxii, 256.

[5] Senter, G., *Z. physikal. Chem.*, 1903, xliv, 257.

the reaction was completed in 2,058 seconds (Fig. 1). The abscissa represents the time; the ordinate the amount of substance decomposed under the influence of the enzyme. Let $A'$ be the time when the enzyme is added, $A$ the time when the reaction starts, and $A'$-$A$ the latency time $T^{\circ}$; $A' - P = T'$; $A' - Q = T''$. If the reaction is monomolecular, the equation of the line $ABC$ will be

$$K = \frac{1}{T} ln \frac{a}{a - x}$$

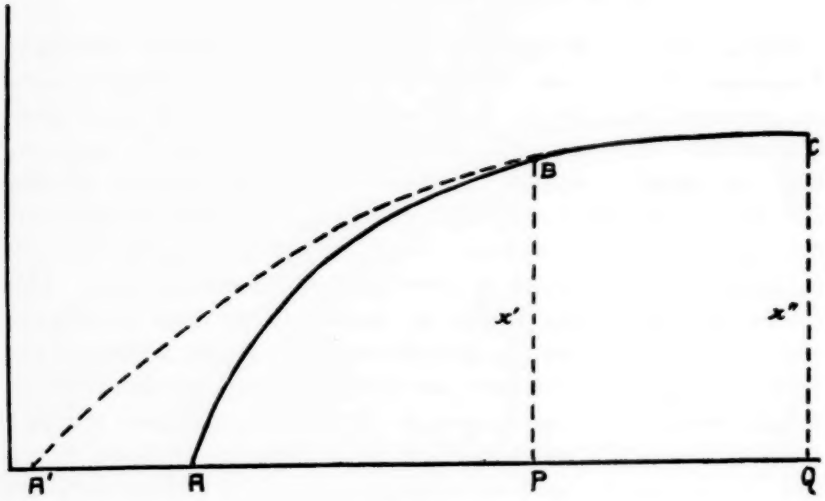


FIG. 1.

in which $K$ represents the reaction velocity, $T$ the time, $a$ the available amount of substrate, and $x$ the amount decomposed. If the reaction is supposed to be polymolecular the following formula will apply.

$$K = \frac{a^n - (a - x)^n}{T . a^n (a - x)^n}$$

in which $n$-1 is the order of the reaction.

The proportion of $T'$ and $T''$ will be

$$\frac{T'}{T''} = \frac{ln \frac{a}{a - x'}}{ln \frac{a}{a - x''}} \qquad (1)$$

if the reaction is monomolecular;

$$\frac{T'}{T''} = \frac{\dfrac{a^n - (a - x')^n}{a^n \cdot (a - x')^n}}{\dfrac{a^n - (a - x'')^n}{a^n \cdot (a - x'')^n}} \qquad (2)$$

if the reaction is polymolecular. On the assumption that $A'$ is the starting point of the reaction we get

$$K' = \frac{1}{T' + T^\circ} ln \frac{a}{a - x}$$

and

$$K'' = \frac{1}{T'' + T^\circ} ln \frac{a}{a - x''} \qquad (3)$$

or, more general

$$K' = \frac{1}{T' + T^\circ} \cdot \frac{a^n - (a - x')^n}{a^n \cdot (a - x')^n}$$

and

$$K'' = \frac{1}{T'' + T^\circ} \cdot \frac{a^n - (a - x'')^n}{a^n \cdot (a - x'')^n} \qquad (4)$$

Equations (1), (2), (3), and (4) give

$$\frac{K'}{K''} = \frac{(T'' + T^\circ)T'}{(T' + T)T''}$$

or, if we take the determinations at regular intervals $T'' = 2T'$ and $\frac{K'}{K''} = \frac{2T' + T^\circ}{2T' + 2T^\circ}$. $K'' > K'$, unless $T^\circ = 0$ or *if there is any latency time the reaction velocity will increase.*

In the work of both Bredig and his coworkers[1, 2] and Senter[5] the formula $K = \frac{1}{t} ln \frac{a}{a - x}$ is used, which gives the reason for the increase in the $K$ values. Waentig and Steche[4] use the modification $K = \frac{1}{t - t_0} ln \frac{a - x_0}{a - x}$ in which $x_0$ is the amount decomposed at a certain time $T$. They do not use the starting point in their calculations. To find out whether the constant $K$ values obtained by these authors

were due to the formula they used, I tested this formula on some experiments of Bredig and von Berneck and of Senter.

| $T$ | $a-x$ | $0.4343\,K$ $\left(\text{From } \log\frac{a}{a-x}.\right)$ | $0.4343\,K$ $\left(\text{From } \log\frac{a-x_0}{a-x}.\right)$ |
|---|---|---|---|
| Values of $K$ obtained (Bredig and von Berneck*). | | | |
| 0 | 19.0 | 0.0147 | |
| 9 | 13.7 | 0.0158 | 0.0168 |
| 14 | 11.4 | 0.0159 | 0.0164 |
| 20 | 9.0 | 0.0162 | 0.0166 |
| Values of $K$ obtained (Bredig and von Berneck†). | | | |
| 0 | 21.5 | | |
| 6 | 19.7 | 0.0063 | |
| 15 | 16.1 | 0.0084 | 0.0098 |
| 30 | 11.5 | 0.0091 | 0.0097 |
| 50 | 6.85 | 0.0099 | 0.0099 |
| Values of $K$ obtained (Senter‡). | | | |
| 0 | 46.4 | | |
| 4.75 | 41.6 | 0.0100 | |
| 15.5 | 32.3 | 0.0102 | 0.0112 |
| 25 | 25.3 | 0.0112 | 0.0106 |
| 40.15 | 17.0 | 0.0114 | 0.0109 |
| 80 | 5.3 | 0.0127 | 0.0104 |

* Bredig, G., and von Berneck, R. M., *Z. physikal. Chem.*, 1899, xxxi, 291.
† Bredig, G., and von Berneck, R. M., *Z. physikal. Chem.*, 1899, xxxi, 297.
‡ Senter, G., *Z. physikal. Chem.*, 1903, xxliv, 280.

It is obvious that the formula $K = \frac{1}{t-t_0} ln \frac{a-x_0}{a-x}$ gives a constant $K$. There certainly are cases in which the use of this formula does not prevent the increase of reaction velocities; *e.g.*, the inversion of cane-sugar.[6] But I am inclined to believe that in most of the cases with catalase this increase was due to the properties of the formula with which the results were calculated.

[6] Henri, V., *Z. physikal. Chem.*, 1902, xxxix, 194.

# ASSOCIATIVE BACTERIAL ACTION IN THE PROPIONIC ACID FERMENTATION.*

By JAMES M. SHERMAN and ROSCOE H. SHAW.

*(From the Research Laboratories of the Dairy Division, United States Department of Agriculture, Washington.)*

(Received for publication, January 13, 1921.)

Among the most interesting problems in bacteriology are those that have to do with the mutual relations of the organisms under natural conditions. Numerous notations of effects, both beneficial and detrimental, of one organism upon another are to be found in bacteriological literature. The beneficial action on a specific organism of the associative growth of another organism is well illustrated by such well known examples as the helpful effect on the tetanus bacillus of associated aerobes; and the increased vigor of the lactic acid fermentation when conducted in impure culture, which has been studied extensively by Marshall and his associates. Antagonisms, aside from those which may be readily explained upon a purely physical or chemical basis, have been noted in many connections. An example of this nature is the recent report of Smith and Smith[1] on the inhibiting action of the paratyphoid organism upon the gas production of *Bacterium coli.*

In the present paper we wish to call attention to a beneficial associative effect of greater magnitude than is frequently observed, and which involves the little studied propionic acid fermentation. The full details of this work will be included in a forthcoming paper on the propionic acid fermentation of lactose.

The propionic acid-producing organism used in these experiments was of the type which has been tentatively designated as *Bacterium acidi propionici* (*d*) and which has been shown to be the essential organism for the production of "eyes" and the characteristic flavor in Swiss cheese.[2] The other organisms employed were *Streptococcus*

* Published with the permission of the Secretary of Agriculture.

[1] Smith, T., and Smith, D. E., *J. Gen. Physiol.*, 1920, iii, 21.

[2] Sherman, J. M., *J. Bact.*, 1921, vi, in press.

*lacticus*, *Lactobacillus casei*, and Cultures 45.3 and 45.4, two non-lactose-fermenting organisms which have not been identified nor subjected to detailed study.

The results of a typical experiment are given in Table I. The cultures were grown for 1 month at 30°C. in a medium containing 5 per cent lactose, 1 per cent peptone, and an excess of calcium carbonate. Similar results have been obtained in many different tests and with other organisms.

From these figures it is seen that this propionic organism is greatly aided in its action by the associated growth of certain other bacteria. In the cases of *Streptococcus lacticus* and *Lactobacillus casei* the

TABLE I.

*Effect of Associative Action.*

| Inoculation. | Propionic acid. |
|---|---|
| | *per cent* |
| Propionic organism alone | 0.081 |
| " " + *Streptococcus lacticus* | 0.470 |
| " " + *Lactobacillus casei* | 0.589 |
| " " + Culture 45.3 | 0.559 |
| " " + " 45.4 | 0.362 |

increased production of propionic acid might be assumed to be due to a greater utilization of the lactic acid produced by these bacteria than of the lactose, since the propionic organism is an active fermenter of lactates. Von Freudenreich and Jensen[3] showed that the volatile acids produced from milk by *Bacterium acidi propionici* (*a*), a feeble lactose fermenter, could be greatly increased by growing the organism in combination with lactic acid-forming organisms. However, our results obtained in other tests in which the fermentable material consisted of calcium lactate instead of lactose also showed a stimulating effect of *Lactobacillus casei* upon the propionic acid-producing organism. In this connection it is interesting to note that certain non-lactose-fermenting bacteria stimulate the propionic acid fermentation of lactose in the same way.

It is hoped that we may return to this problem at some future time in an effort to throw some light on the nature of the associative effect.

[3] von Freudenreich, E., and Jensen, O., *Landw. Jahrb.*, 1906, xx, 320.

# THE PHYSIOLOGICAL ZERO: AN EXPLANATION OF THE DEPARTURE FROM THE LINEAR GRAPH OF REACTION RATE VALUES AT THE LOWER TEMPERATURES.*

By JOSEPH KRAFKA, Jr.

With the Collaboration of R. P. Stevens and David F. Barrow.

(*From the Zoological Laboratory of the University of Georgia, Athens.*)

(Received for publication, January 25, 1921.)

Considerable controversy has arisen between investigators dealing with the influence of temperature upon physiological reaction rates. Some reactions are decidedly exponential, others clearly linear functions of the temperature. To the first class belong the results of Snyder (1913), on the rate of beat of the mammalian heart and those of Loeb and Northrop (1917) on the length of life of *Drosophila*. The work of Sanderson (1910), Peairs (1914) and others on insect development and that of Krogh (1914) on general metabolism establish the latter relationships.

Marked deviations from the linear curves have been noted at the upper and lower temperatures, making necessary the qualifying clause that the linear relations only held between the normal limits of growth. The departures at the upper temperatures have received an explanation. It is generally conceded that the linear is a modified exponential whose secondary factor increases more rapidly than the primary one at the higher temperatures.

The departures at the lower temperatures have only been noted in connection with methods for the calculation of the physiological zero. Thus if we were to apply Peairs' method for the determination of the physiological zero to the development of *Drosophila melanogaster* it would come at about 8°C. in the work of Loeb and Northrop (1917), and at 10°C. in my own experiments (Krafka (1919–20)).

* Contribution from the Zoological Laboratory of the University of Georgia, No. 1.

Yet Plough reared larvae and pupae at 5°C. Similar deviations are apparent in all of Krogh's curves.

These deviations at the lower temperatures further support the hypothesis that the linear curve

$$y \infty x$$

is in reality a modified exponential curve

$$y \infty 2^x$$

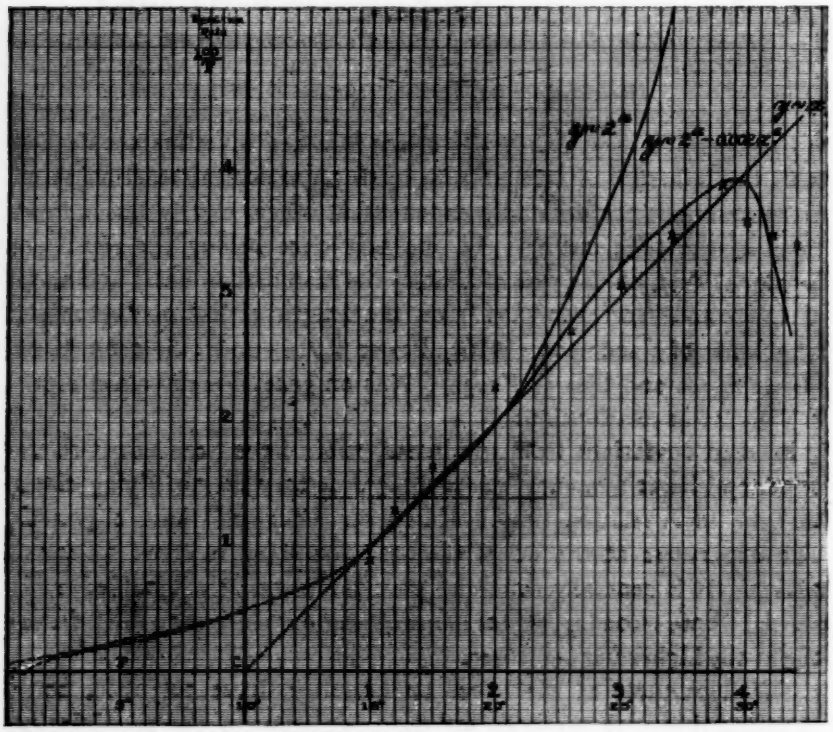

FIG. 1. Curves representing the relations between the temperature and the rate of development of the fruit fly, *Drosophila melanogaster*. The experimental points $x$ fall along the linear curve $y \infty x$ between certain limits. Projecting this curve to the base line the physiological zero of Peairs is obtained at 10°C. The point P represents an observation establishing development below this by Plough. The curve $y \infty 2^x$ is a theoretical van't Hoff curve involving two experimental values, while the modified exponential is shown as the middle one at each end.

for under these conditions they are normal and expected, since they represent only the continuation of the primary exponential.

Dr. R. P. Stephens and Dr. David F. Barrow have supplied me with an empirical formula from data on the rate of development of the fruit fly, *Drosophila melanogaster*, which fulfills the conditions for the flattening of a primary exponential into a linear with a sharp bend at the upper end.

$$y \propto 2^x - 0.002x^6$$

This corrective factor at the same time breaks the primary exponential at the lower temperatures so that a theoretical zero becomes possible.

While any number of mathematical functions could be devised whose graphs would fit the experimental points fairly well, the latter formula stated in general terms should have some practical value.

$$y \propto A^x - Bx^n$$

where $A$ represents van't Hoff's constant, $B$ and $n$ calculated values to represent the divergence between the linear and exponential at the uppermost points.

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# COMPARATIVE STUDIES ON RESPIRATION.

## XVII. Decreased Respiration and Recovery.

By O. L. INMAN.

*(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)*

(Received for publication, March 17, 1921.)

In a previous paper the writer[1] has shown that exposure to hypotonic or hypertonic solutions may greatly lower the production of carbon dioxide by *Laminaria agardhii*. The object of the present investigation was to determine whether the respiration would become normal when the plant was replaced in sea water. The work was done at Woods Hole, where material could be obtained under the most favorable conditions.

The normal rate of respiration was determined (in the manner previously described) and the tissue was exposed to the hypertonic or hypotonic solution for a definite time; it was then removed and its respiration was measured.[2] The piece of tissue was then replaced in running sea water. At varying intervals it was removed and the rate of respiration was determined. As in the previous investigation[1] these determinations were made in van't Hoff's solution in order to avoid the buffer effect of the sea water.

Fig. 1 shows that in strongly hypertonic sea water respiration steadily decreases and that the degree of recovery depends on the length of the exposure. After an exposure of five minutes recovery is practically complete: as the period of exposure is lengthened recovery is less and less complete and when the exposure amounts to 20 minutes there is no recovery whatever.

It is evident that the upper curves dip slightly after the tissues are returned to normal sea water. This is not seen in the experiments with hypotonic solutions and isotonic sodium chloride and may

[1] Inman, O. L., *J. Gen. Physiol.*, 1921, iii, 533.

[2] The measurement of respiration was always made with the tissues in the dark.

perhaps be explained as a purely mechanical disturbance due to the great differences in osmotic pressure as the result of which the tissue must remain about 30 minutes in normal sea water before the readjustment is complete.

Fig. 2 shows that the effects of strongly hypotonic sea water are similar to those of strongly hypertonic, except that the lowering of respiration is not so pronounced. Here also we observe all degrees of recovery depending on the length of exposure to the solution.

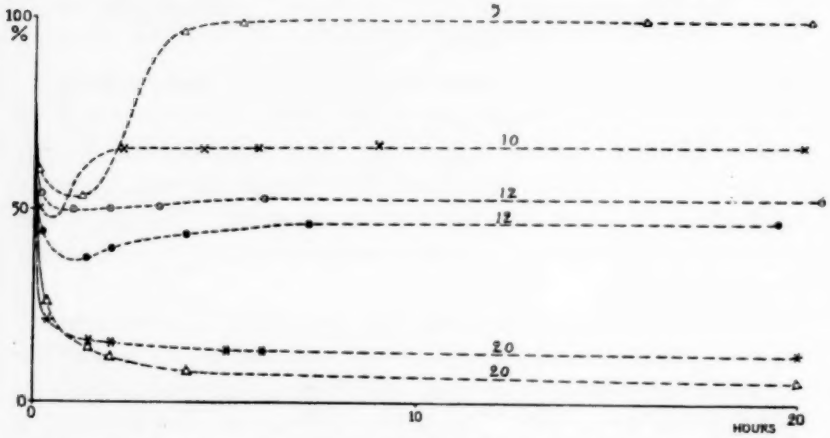


FIG. 1. Curves showing rate of respiration of *Laminaria* (expressed as per cent of the normal). The normal rate represents a change from pH 7.78 to 7.36 in from 1½ minutes to 2 minutes, depending upon the amount of material used. The solid lines show rate of respiration while tissue was exposed to hypertonic sea water (sp. gr. 1.130, $\Delta = -9.37$ approximately). The dotted lines show stages of recovery after the tissue was put back in normal sea water. Each curve represents a typical experiment. The figure attached to each recovery curve denotes the time (in minutes) of exposure to the solution of hypertonic sea water; thus the uppermost curve represents recovery, after an exposure of 5 minutes.

The question naturally arises as to what happens when these pieces of tissue are kept longer than 20 hours in running sea water. This was carefully investigated and it was found that every piece of tissue which had been exposed to hypertonic or hypotonic sea water died and disintegrated much sooner in running sea water than the normal piece of tissue kept in the same vessel and treated in the same manner except that it was not exposed to hypertonic or hypotonic solutions.

Pieces of tissue that showed complete recovery were found to live longest, as expected, but none of these remained normal more than seven days while the untreated showed a normal rate of respiration after eighteen days. Those pieces that showed partial recovery were marked by a fall in the rate, usually at the end of three days, but in a few cases not until the end of five days. Where there was no recovery the pieces turned green and soon disintegrated.

A series of experiments was made in which pieces of *Laminaria* were placed in isotonic sodium chloride (0.52 M for Woods Hole sea water) and the respiration measured at the end of an hour. The

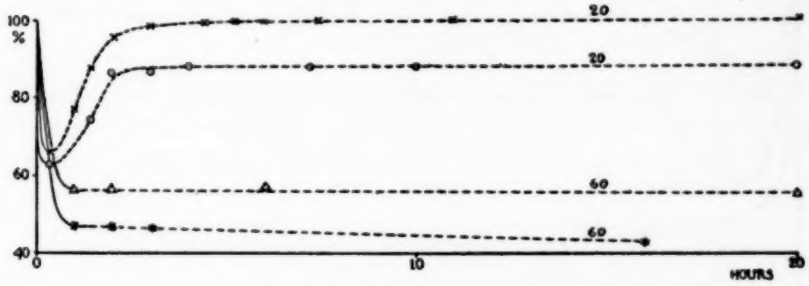


FIG. 2. Curves showing rate of respiration of *Laminaria* (expressed as per cent of the normal). The normal rate represents a change from pH 7.78 to 7.36 in from 1½ to 2 minutes, depending upon the amount of material used. The solid lines show rate of respiration while tissue was exposed to hypotonic sea water (sp. gr. 1.0023, $\Delta = -0.187$). The dotted lines show stages of recovery after the tissue was put back in normal sea water. Each curve represents a typical experiment. The figure attached to each recovery curve denotes the time (in minutes) of exposure to the solution of hypotonic sea water.

pieces were then returned to running sea water and at intervals shown by the points on dotted lines of Fig. 3, they were removed and the rate of respiration determined.

In this case some pieces of tissue were more affected than others by the same length of exposure (due to the thickness of the fronds, the temperature of the experiment, etc.), and it is noticeable that the greater the lowering of the respiration the less complete the recovery. Here also it was found that the treated pieces of tissue lived but a short time in running sea water as compared with the normal pieces.

It is therefore evident that in all these experiments recovery may be incomplete and even where the tissue is kept for days under the most favorable circumstances there is no tendency in these cases for the recovery to complete itself. This offers a striking parallel to the experiments recently described by Osterhout[3] in which all degrees of recovery (depending upon the length of exposure) were observed when *Laminaria* was placed for a time in 0.52 M sodium chloride (and in other solutions)[4] and then replaced in sea water. In this case electrical conductivity was used as a criterion of recovery.

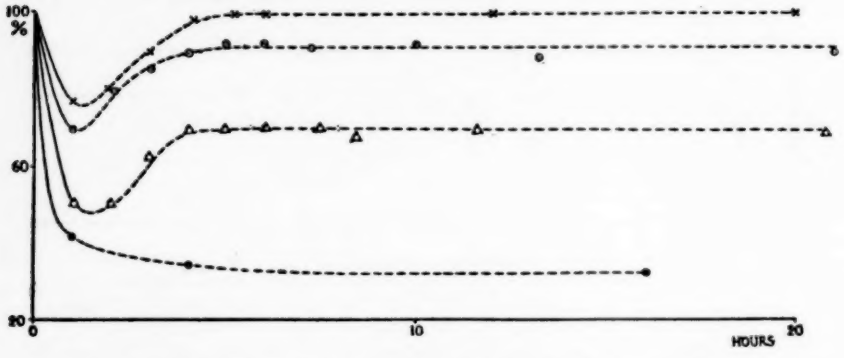


FIG. 3. Curves showing rate of respiration of *Laminaria* (expressed as per cent of the normal). The normal rate represents a change from pH 7.78 to 7.36 in from 1½ to 2 minutes, depending upon the amount of material used. The solid lines show rate of respiration during one hour of exposure to isotonic sodium chloride (0.52M for Woods Hole sea water). The dotted lines show stages of recovery after the tissue was put back in normal sea water. Each curve represents a typical experiment.

It is possible that when recovery is incomplete none of the cells are killed but that their respiration is permanently decreased. It is also possible that some cells continue to respire normally while others are killed and that it is the death of these cells which prevents complete recovery. This question must remain for future investigation.

[3] Osterhout, W. J. V., *J. Gen. Physiol.*, 1920, iii, 15.

[4] Unpublished experiments by Osterhout on the electrical conductivity of *Laminaria* show incomplete recovery after exposure to hypo- and hypertonic solutions.

# DONNAN EQUILIBRIUM AND THE PHYSICAL PROPERTIES OF PROTEINS.

## I. Membrane Potentials.

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### I. INTRODUCTION.

The different physical properties of proteins, such as osmotic pressure, swelling, and viscosity, vary in a similar way under the influence of changes in the concentration of hydrogen ions or under the influence of neutral salts or under the influence of the valency of the ion in combination with the protein. This fact suggests that the variations of these three physical properties may have a common cause, so that if this cause were known for one of these properties it would, perhaps, be known for all of them. The clues tentatively suggested have thus far either proved wrong or inaccessible to quantitative tests.

In the last paper[1] the writer showed that a measurable potential difference is found when we separate a protein solution from a watery solution (free from protein) by a collodion membrane. When the protein in solution is a protein-acid salt (*e.g.*, gelatin chloride) the protein solution is positive and the water is negative; when the protein exists in the form of a metal proteinate the protein solution is negative and the watery solution positive. The turning point seems to lie at the isoelectric point. Quantitative measurements of these P.D. between gelatin chloride solutions and the outside watery solution with which the gelatin chloride solution was in osmotic equilibrium revealed the fact that these potential differences varied in a similar way as the osmotic pressure, the swelling, or the viscosity

[1] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 557.

when a neutral salt was added or when the hydrogen ion concentration was altered or when the valency of the ion in combination with the protein changed. This in itself would have meant only the addition of another property varying in the same characteristic way as osmotic pressure, swelling, or viscosity if it had not been for the fact that it was possible to correlate the origin of this new property with Donnan's membrane equilibrium. Donnan's membrane equilibrium[2] is established when a membrane separates two solutions of electrolytes one of which has one ion for which the membrane is impermeable, while all the other ions can diffuse through the membrane. It is not necessary that this non-diffusible ion should be a colloid, it may just as well be a crystalloid; all that is required is that it be impossible for this ion to diffuse through the membrane. The protein ions generally satisfy this condition and a collodion membrane properly prepared is a membrane impermeable to a protein ion.

When equilibrium is established in such a system the distribution of the ions is not the same on both sides of the membrane and from thermodynamic considerations Donnan was able to develop the equations for the relative concentration of the different ions on opposite sides of the membrane at equilibrium. When a collodion bag filled with a 1 per cent gelatin chloride solution is dipped into a beaker containing a solution of HCl (without gelatin) of the same pH as that of the gelatin solution, the concentration of the hydrochloric acid becomes greater outside than inside the gelatin solution. The Donnan equilibrium demands that free acid be expelled from the gelatin solution into the outside solution and this actually occurs.[3]

Procter[4] has proposed an osmotic theory of the swelling of gelatin chloride on the assumption that the swelling is a purely osmotic phenomenon. He deduces from Donnan's theory that at the point

[2] Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572. Donnan, F. G., and Harris, A. B., *J. Chem. Soc.*, 1911, xcix, 1554. Donnan, F. G., and Garner, W. E., *J. Chem. Soc.*, 1919, cxv, 1313.

[3] This is possible on account of the fact that a gelatin chloride solution always contains free HCl and that there seems to be a chemical equilibrium inside the gelatin chloride solution between the free HCl, the gelatin chloride, and the non-ionogenic gelatin.

[4] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

of equilibrium the relative distribution of HCl inside and outside the solid block of gelatin chloride is determined by the following equation:

$$x^2 = y\,(y + z)$$

where $x$ is the concentration of H ions (and of Cl ions) outside the gelatin, $y$ the concentration of the H (and Cl) ions of the free HCl inside the gelatin, and $z$ the concentration of the Cl ions in combination with the gelatin ions. Since all quantities are positive, it follows that $x$ must be greater than $y$; *i.e.*, the concentration of free HCl in the outside solution must be greater than in the gel. The writer has shown that exactly the same happens when we separate a solution of gelatin chloride from pure water by a collodion membrane.

This difference in the concentration of acid on the two sides of the membrane leads to a difference in P.D. at the boundary of the membrane.[5]

This can be proved by the fact that it is possible to calculate the P.D. of the system

$$- \left| \begin{array}{c} \text{outside solution} \\ \text{(free from gelatin)} \\ \text{concentrated HCl} \end{array} \right| \begin{array}{c} \text{collodion} \\ \text{membrane} \end{array} \left| \begin{array}{c} \text{inside solution} \\ \text{(gelatin chloride)} \\ \text{dilute HCl} \end{array} \right| +$$

with a fair degree of accuracy on the basis of Nernst's well known logarithmic formula from the difference of the hydrogen ion concentration on the opposite sides of the membrane. On the basis of Nernst's formula for concentration cells the P.D. is at a temperature of 24°C. $0.059 \log \frac{C_1}{C_2}$, where $C_1$ is the concentration of hydrogen ions inside the gelatin solution and $C_2$ the hydrogen ion concentration outside the gelatin solution. Hence we may substitute the value pH inside minus pH outside for the value $\log \frac{C_1}{C_2}$. (The term pH inside means the pH in the gelatin solution and the term pH outside means the pH in the outside solution with which the gelatin solution is in equilibrium.) This paper intends to show that if we multiply

[5] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 247.

the value of pH inside minus pH outside by 59 we can calculate with a fair degree of accuracy the P.D. observed at 24°C. in millivolts when equilibrium between the gelatin chloride solution and the outside solution is established.

## II. METHODS.

The potential differences were determined with the aid of a Compton electrometer giving a deviation of about 2 mm. on the scale for 1 millivolt at a distance of about 2 m. The gelatin solution was inside the collodion bag closed with a rubber stopper through which a funnel was introduced; the funnel was filled high enough with liquid to permit the electrode to dip into the gelatin solution. The collodion bag containing the solution of gelatin-acid salt (*e.g.*, gelatin chloride) dipped into the water with which the gelatin solution was in equilibrium. The water, therefore, contained always free acid and in certain experiments also salt solution when the nature of the experiment demanded this. The second electrode was introduced into this outside watery solution. Calomel electrodes with saturated KCl solution were used and the saturated KCl solutions were brought into contact with the outside and inside solutions through glass tubes. The ends of these tubes which dipped into the inside and outside solutions were drawn out into capillaries and bent upwards to prevent the influence of gravity on the diffusion of the saturated KCl solution into the inside and outside solutions. The only potential differences existing in this system were those on the opposite sides of the membrane.

## *III. The Influence of Neutral Salts on the Potential Difference between Gelatin Chloride Solutions and Outside Solutions.*

1 gm. of isoelectric gelatin was made into a 1 per cent solution by either dissolving it in $H_2O$ or in a solution of NaCl differing in molecular concentration from M/4,096 to 1 M. To every solution so much HCl was added that the pH of the solution was 3.5. Collodion bags of a volume of about 50 cc. were filled with this solution, each collodion bag being connected with a glass tube serving as manometer, as described in preceding publications. These collodion bags

were dipped into beakers containing 350 cc. of HCl solution of pH 3.0. The HCl solutions in the beakers were made up in NaCl solutions of different concentrations and the concentration of the NaCl solution in the beaker was at the beginning of the experiment always identical with the concentration of the NaCl in the gelatin solution inside the collodion bag. The final measurements were made after 18 hours when the osmotic and the membrane equilibria were established. The osmotic pressure was a maximum (about 425 mm. water) in the gelatin chloride solution free from salt, and the osmotic pressure was the lower the higher the concentration of the salt added. This effect is represented in the upper curve of Fig. 1. The abscissae are the concentration of the NaCl solution and the ordinates the osmotic pressure. The curve shows that the osmotic pressure drops very rapidly with the increase in the concentration of NaCl.

The potential differences at the boundary of the inside and outside solutions were measured with a Compton electrometer as described and the values found are plotted on the second upper curve in Fig. 1. The scale for the ordinates was selected in such a way as to make the osmotic pressure ordinate and the ordinate for the P.D. coincide for a M/4,096 NaCl solution. The reader will notice that the two curves for osmotic pressure and P.D. coincide practically throughout which signifies that the P.D. and the osmotic pressure of the gelatin chloride solution undergo a similar depression under the influence of a neutral salt like NaCl.

We stated in the last paper that the observed P.D. is always equal to the P.D. calculated on the assumption that at equilibrium the P.D. is due to the difference in the pH inside and outside the collodion bag. The pH inside and outside were measured electrometrically and the results are contained in Table I.

On the assumption stated above we can calculate the P.D. in millivolts by multiplying the value pH inside minus pH outside by 59, and the values so obtained should agree with the observed P.D. Table II shows that this is true.

The two lower curves of Fig. 1 give the depressing effect of different concentrations of $Na_2SO_4$ on the osmotic pressure and on the P.D. of a 1 per cent gelatin chloride solution of pH 3.5. Everything was the same as in the preceding experiment, except that $Na_2SO_4$ was sub-

stituted for NaCl. The two lower curves in Fig. 1 show that the depressing effect of $Na_2SO_4$ on the osmotic pressure and the P.D. of a gelatin chloride solution is very similar and, moreover, that the depressing effect of $Na_2SO_4$ on both properties is more than twice (in reality nearly eight times) as great as that of NaCl. This influence

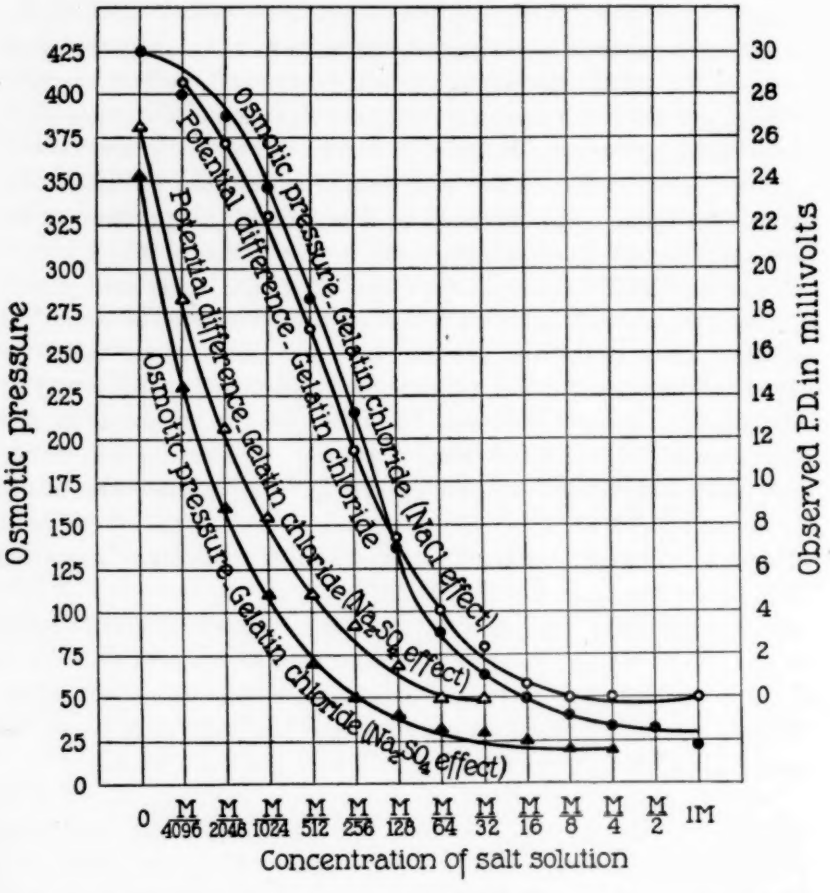


FIG. 1. Depressing effect of NaCl and $Na_2SO_4$ on osmotic pressure and P.D. of gelatin chloride solutions. Abscissae represent the concentration of salt solution, ordinates the osmotic pressure and P.D. in millivolts respectively. The depressing effect of neutral salts on osmotic pressure and P.D. is similar but not identical.

TABLE I.

*Influence of Concentration of NaCl on pH Inside Minus pH Outside at Equilibrium.*

| | Concentration of NaCl. | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | M/4,096 | M/2,048 | M/1,024 | M/512 | M/256 | M/128 | M/64 | M/32 | M/16 | M/8 | M/4 | M/2 | 1 M |
| pH inside..... | 3.60 | 3.55 | 3.52 | 3.46 | 3.41 | 3.36 | 3.31 | 3.27 | 3.22 | 3.30 | 3.32 | 3.32 | 3.28 | 3.19 |
| pH outside..... | 3.09 | 3.08 | 3.09 | 3.11 | 3.13 | 3.16 | 3.20 | 3.20 | 3.18 | 3.28 | 2.30 | 3.30 | 3.26 | 3.19 |
| pH inside minus pH outside... | 0.51 | 0.47 | 0.43 | 0.35 | 0.28 | 0.20 | 0.11 | 0.07 | 0.04 | 0.02 | 0.02 | 0.02 | 0.02 | 0.00 |

TABLE II.[6]

*Depressing Effect of NaCl on P.D. at Equilibrium.*

| Molecular concentration of NaCl. | P. D. observed. | P. D. calculated. |
|---|---|---|
| | *millivolts* | *millivolts* |
| 0 | | +29.9 |
| M/4,096 | +28.6 | +27.6 |
| M/2,048 | +25.7 | +25.2 |
| M/1,024 | +22.3 | +20.6 |
| M/512 | +17.1 | +16.4 |
| M/256 | +11.4 | +11.7 |
| M/128 | + 7.4 | + 6.5 |
| M/64 | + 4.0 | + 4.1 |
| M/32 | + 2.3 | + 2.3 |
| M/16 | + 0.6 | + 1.2 |
| M/8 | 0.0 | + 1.2 |
| M/4 | 0.0 | + 1.2 |
| M/2 | − 1.7 | + 1.2 |
| 1 M | 0.0 | 0.0 |

[6] The sign of the observed P.D. was apparently, but not in reality, the reverse of the sign of the calculated P.D. This was due to the difference in the arrangement of the hydrogen electrodes in both cases. In the "observed" P.D. the membrane (serving as a hydrogen electrode) was between the concentrated and dilute HCl, while in the "calculated" values the P.D. was obtained from the potentiometric determinations of the pH. In this latter case the two hydrogen electrodes were separated by a concentrated and a dilute solution. The "observed" P.D. was hence between two solutions of different concentrations while in the "calculated" values we measured the P.D. between two electrodes. In our tables the apparent (but not real) reversal of sign due to the difference in arrangement of the hydrogen electrodes in the two cases is corrected.

of the valency of anion in the case of gelatin-acid salt has been discussed in preceding papers. The question now arises: Does the value of the difference in the pH inside minus pH outside vary similarly as the values of the observed P.D. and the observed osmotic pressure, and do we get values approximating the observed P.D. if we multiply the value pH inside minus pH outside by 59? Table III gives the values for pH inside and outside and also the difference, pH inside minus pH outside, at the point of equilibrium (*i.e.*, after 18 hours).

The values for the P.D. calculated by multiplying the values for pH inside minus pH outside by 59 agree remarkably well with the observed values for P.D.

TABLE III.

*Influence of Concentration of $Na_2SO_4$ on pH Inside Minus pH Outside at Equlibrium.*

| | Concentration of $Na_2SO_4$. | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | M/4,096 | M/2,048 | M/1,024 | M/512 | M/256 | M/128 | M/64 | M/32 | M/16 | M/8 | M/4 |
| pH inside | 3.54 | 3.41 | 3.35 | 3.32 | 3.29 | 3.30 | 3.33 | 3.38 | 3.41 | 3.41 | 3.37 | 3.29 |
| pH outside | 3.07 | 3.12 | 3.14 | 3.17 | 3.20 | 3.24 | 3.30 | 3.35 | 3.38 | 3.38 | 3.36 | 3.28 |
| pH inside minus pH outside | 0.47 | 0.29 | 0.21 | 0.15 | 0.09 | 0.06 | 0.03 | 0.03 | 0.03 | 0.03 | 0.01 | 0.01 |

We will consider as a third case the influence of a salt of the type $CaCl_2$ on the osmotic pressure and the P.D. of a gelatin chloride solution. It had been shown in a preceding paper that the depressing effect of $CaCl_2$ is always about twice as great as that of an equimolecular concentration of NaCl and that hence the whole effect of $CaCl_2$ is due exclusively to the Cl ion. Fig. 2 shows that the depressing effects of $CaCl_2$ on the osmotic pressure and the P.D. run closely parallel and that the depressing effect of $CaCl_2$ on P.D. is about the same as that of a NaCl of twice the molecular concentration. Table V gives the influence of $CaCl_2$ on the values of pH inside minus pH outside, and shows that the agreement between the observed and the calculated P.D. is quite close.

It follows from these experiments, first, that the curves representing the influence of neutral salts on P.D. are similar to the curves representing the influence of the same salts on the osmotic pressure of

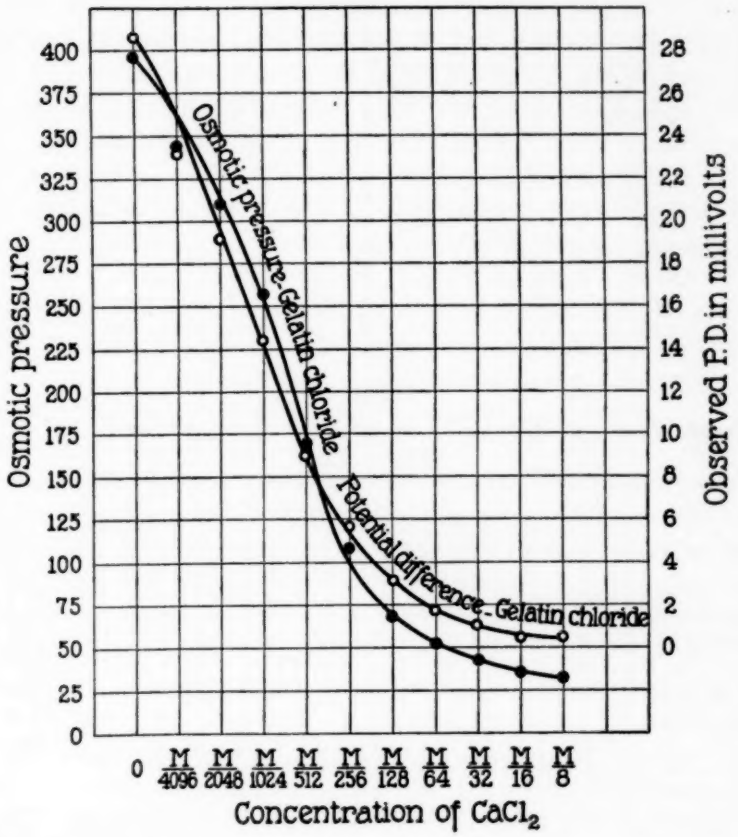


FIG. 2. Similarity of depressing effect of $CaCl_2$ on osmotic pressure and P.D. of gelatin chloride solution.

the same gelatin chloride solutions; and second, that the P.D. calculated by multiplying the values of pH inside minus pH outside by 59 agrees quite closely with the observed P.D.

TABLE IV.

*Depressing Effect of $Na_2SO_4$ on P.D. at Equilibrium.*

| Molecular concentration of $Na_2SO_4$. | P. D. observed. | P. D. calculated. |
|---|---|---|
| | *millivolts* | *millivolts* |
| 0 | +26.5 | +27.6 |
| M/4,096 | +18.6 | +17.0 |
| M/2,048 | +12.5 | +12.3 |
| M/1,024 | + 8.4 | + 8.8 |
| M/512 | + 4.7 | + 5.3 |
| M/256 | + 3.4 | + 3.5 |
| M/128 | + 1.5 | + 1.7 |
| M/64 | 0.0 | + 1.7 |
| M/32 | 0.0 | + 1.7 |
| M/16 | 0.0 | + 1.7 |
| M/8 | 0.0 | + 0.6 |
| M/4 | 0.0 | + 0.6 |

TABLE V.

*Influence of Concentration of $CaCl_2$ on pH Inside Minus pH Outside and on P.D. at Equilibrium.*

| | Concentration of $CaCl_2$. | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | M/4,096 | M/2,048 | M/1,024 | M/512 | M/256 | M/128 | M/64 | M/32 | M/16 | M/8 |
| pH inside..... | 3.55 | 3.45 | 3.41 | 3.36 | 3.30 | 3.28 | 3.26 | 3.25 | 3.25 | 3.25 | 3.22 |
| pH outside.... | 3.05 | 3.06 | 3.09 | 3.12 | 3.15 | 3.17 | 3.20 | 3.22 | 3.24 | 3.24 | 3.22 |
| pH inside minus pH outside..... | 0.50 | 0.39 | 0.32 | 0.24 | 0.15 | 0.11 | 0.06 | 0.03 | 0.01 | 0.01 | 0.0 |
| P.D. calculated (millivolts).. | +29.5 | +23.0 | +18.9 | +14.1 | +8.8 | +6.5 | +3.5 | +1.8 | +0.6 | +0.6 | 0.0 |
| P.D. observed (millivolts).. | +28.6 | +23.4 | +19.2 | +14.5 | +9.1 | +5.7 | +3.1 | +1.8 | +1.1 | +0.5 | +0.5 |

## *IV. Influence of Neutral Salts on the Potential Differences between Solid Gelatin and Outside Solution.*

It is well known that the addition of neutral salt depresses the swelling of solid gelatin chloride or of any gel with a pH different from that of the isoelectric point. This is intelligible on the basis of

Procter's osmotic theory of swelling which assumes that the swelling of a gel is due to the osmotic pressure inside the gel, the surface of the gel acting as a membrane impermeable to gelatin ions but permeable to the H or Cl or salt ions. On this assumption we should expect the establishment of a Donnan equilibrium between the liquid inside the gel and the outside liquid, and Procter has proved that this is the case. We should also expect a P.D. at the surface of the gel between the solution inside and outside the gel, due to the Donnan equilibrium, and we should, moreover, expect that the P.D. could be calculated from the value of pH inside minus pH outside as described for the experiments on osmotic pressure. This expectation is confirmed except that the results are liable to be less regular than in the case of the osmotic pressure experiments. This is probably due to accidental errors, one being possibly that some of the gel dissolves in the outside solution so that the outside solution is no longer free from gelatin. Such an error does not exist when we separate a gelatin solution from the outside solution by a collodion membrane.

Our method was as follows. 1 gm. of powdered gelatin of pH 7.0 was brought to the isoelectric point by treatment with M/128 acetic acid and subsequent washing with cold $H_2O$ as described in previous papers. The doses of powdered wet isoelectric gelatin were then put into 200 cc. of M/128 HCl made up in different concentrations of NaCl varying from 0 to M/8, and the degree of swelling was measured in terms of the height of a column of the powdered gelatin in 100 cc. graduates after equilibrium was established (after two hours). From this the volume of the isoelectric gelatin was deducted. Fig. 3 shows that the swelling diminished with the quantity of salt added. The mass was put on a filter and allowed to drain thus separating the gelatin from the supernatant liquid. The gelatin was then melted and its pH determined electrometrically. This gave us the pH inside, and by determining the pH of the outside solution the values of the pH inside minus pH outside were ascertained. The liquid gelatin was then poured into cylinders with a small bent side tube attached, which was also filled with gelatin. The gelatin was then cooled and allowed to set to a jelly. The P.D. between the solid jelly and the outside solution was then determined by pushing one electrode into the gel while the other electrode was introduced into the

beaker containing the outside solution. The latter solution was brought into contact with the gelatin in the side tube, the latter

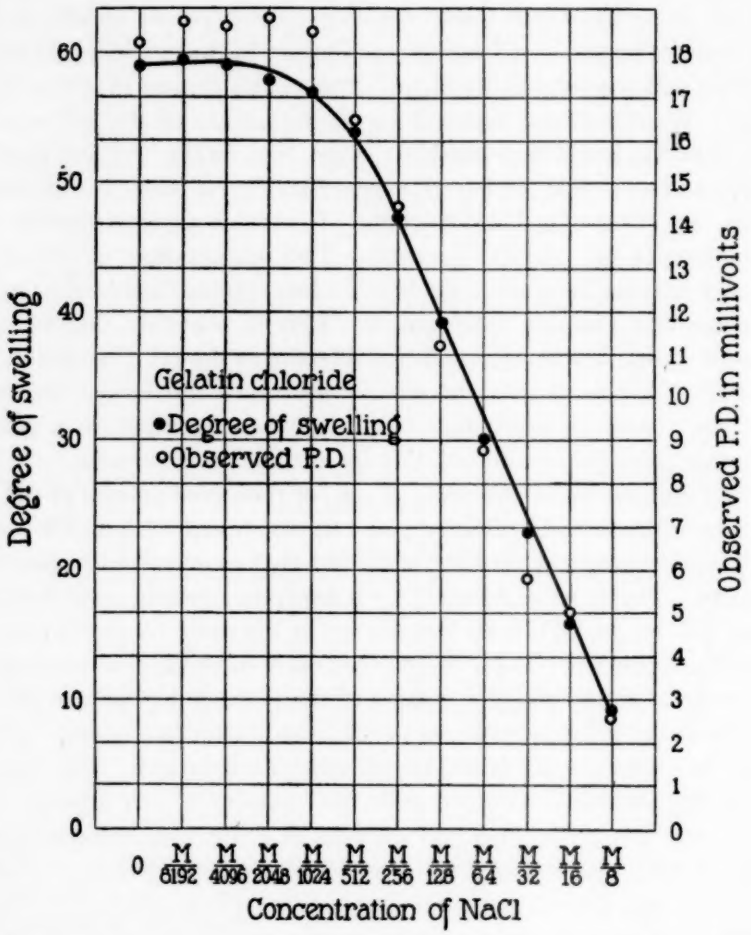


FIG. 3. Similarity of depressing effect of NaCl on swelling and P.D. of solid gelatin chloride.

dipping into the beaker. Table VI gives a comparison of the observed P.D. and those calculated by multiplying the values pH inside minus pH outside by 59. The agreement is satisfactory.

TABLE VI.

*Influence of Concentration of NaCl on pH Inside Minus pH Outside and on P.D. of Solid Gelatin Chloride at Equilibrium.*

| | Concentration of NaCl. | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | M/8,192 | M/4,096 | M/2,048 | M/1,024 | M/512 | M/256 | M/128 | M/64 | M/32 | M/16 | M/8 |
| pH inside | 2.71 | 2.70 | 2.69 | 2.69 | 2.70 | 2.69 | 2.65 | 2.61 | 2.57 | 2.56 | 2.54 | 2.50 |
| pH outside | 2.38 | 2.38 | 2 37 | 2.37 | 2.38 | 2.38 | 2.39 | 2.40 | 2.42 | 2.43 | 2.45 | 2.46 |
| pH inside minus pH outside | 0.33 | 0.32 | 0.32 | 0.32 | 0.32 | 0.31 | 0.26 | 0.21 | 0.15 | 0.13 | 0.09 | 0.04 |
| P.D. calculated (millivolts) | +19.4 | +18.8 | +18.8 | +18.8 | +18.8 | +18.2 | +15.3 | +12.4 | +8.8 | +7.6 | +5.3 | +2.4 |
| P.D. observed (millivolts) | +18.2 | +18.7 | +18.6 | +18.8 | +18.5 | +16.4 | +14.3 | +11.2 | +8.8 | +5.8 | +5.0 | +2.6 |

The same experiment was repeated with $Na_2SO_4$ as a salt instead of with NaCl. A comparison of Fig. 4 with Fig. 3 shows that $Na_2SO_4$ depresses the swelling of gelatin chloride more powerfully than NaCl

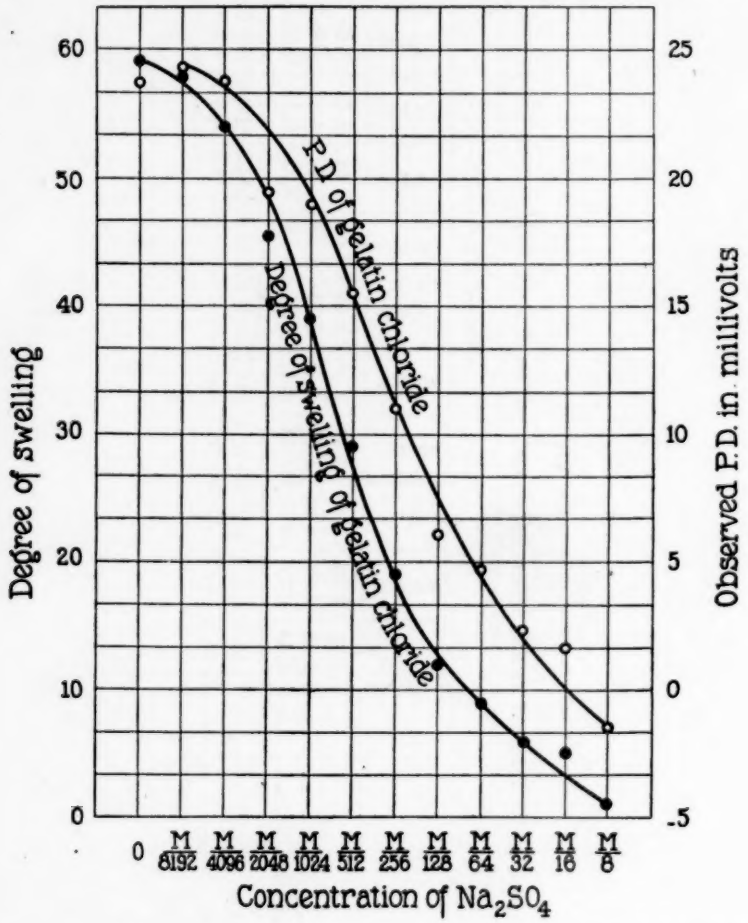


FIG. 4. Similarity of depressing effect of $Na_2SO_4$ on swelling and P.D. of solid gelatin chloride.

and that it depresses also the P.D. more powerfully than is done by NaCl. Table VII gives the pH inside minus pH outside, and a comparison of the P.D. calculated and observed. The agreement is again satisfactory.

TABLE VII.

*Influence of Concentration of $Na_2SO_4$ on pH Inside Minus pH Outside and on P.D. of Solid Gelatin Chloride at Equilibrium.*

| | Concentration of $Na_2SO_4$. | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | m/8,192 | m/4,096 | m/2,048 | m/1,024 | m/512 | m/256 | m/128 | m/64 | m/32 | m/16 | m/8 |
| pH inside | 2.69 | 2.69 | 2.70 | 2.70 | 2.70 | 2.67 | 2.67 | 2.67 | 2.68 | 2.75 | 2.82 | 2.88 |
| pH outside | 2.40 | 2.38 | 2.37 | 2.38 | 2.42 | 2.43 | 2.48 | 2.53 | 2.61 | 2.71 | 2.82 | 2.92 |
| pH inside minus pH outside | 0.29 | 0.31 | 0.33 | 0.32 | 0.28 | 0.24 | 0.19 | 0.14 | 0.07 | 0.04 | 0.00 | −0.04 |
| P.D. calculated (millivolts) | +17.0 | +18.3 | +19.5 | +19.0 | +16.5 | +14.1 | +11.2 | +8.2 | +4.1 | +2.5 | 0.0 | −2.5 |
| P.D. observed (millivolts) | +23.7 | +24.3 | +23.7 | +19.5 | +19.0 | +15.5 | +11.0 | +6.0 | +4.8 | +2.4 | +1.8 | −1.5 |

We therefore come to the conclusion that the depressing influence of the neutral salts on the P.D. between solid gelatin and a surrounding aqueous solution runs parallel to the depressing effect of the same salts on the swelling of gelatin and that the P.D. can be calculated with the aid of Nernst's formula, on the assumption that the difference in the hydrogen ion concentration inside the gel and in the outside solution determines the P.D.

### *V. The Influence of the Hydrogen Ion Concentration of Gelatin Solutions on the P.D.*

The osmotic pressure of 1 per cent solutions of originally isoelectric gelatin varies with the pH of the gelatin solution and with the valency of the ion in combination with the gelatin. This is illustrated in Fig. 5 where the ordinates represent the observed osmotic pressures of 1 per cent solutions of gelatin chloride, gelatin phosphate, gelatin oxalate, and gelatin sulfate. The osmotic pressure rises steeply as soon as the pH becomes less than 4.7, reaching a maximum at pH of about 3.6, and then drops steeply with a further decline of pH. Moreover, it is obvious that the curves for gelatin chloride and phosphate, both possessing a monovalent anion, are identical, that the curve for gelatin oxalate, which has mainly a monovalent anion at the pH under discussion, is almost but not quite as high as that for gelatin chloride, but that the curve for gelatin sulfate (possessing a bivalent anion) is only about half as high as that for gelatin chloride. The P.D. of 1 per cent solutions of these four salts contained in collodion bags were measured against outside aqueous solutions (without gelatin) after equilibrium was reached (*i.e.* after about 18 hours). The bags contained about 50 cc. of the gelatin solution while the beaker contained 350 cc. $H_2O$ with so much acid that the pH of the water was at the beginning of the experiment always identical with the pH of the gelatin solution; and for the outside solution the same acid was used as for the gelatin solution. Fig. 6 gives the curves for the value of the P.D. observed. The following points of similarity between the two sets of curves for osmotic pressure (Fig. 5) and P.D. (Fig. 6) are noticeable. Both sets of curves rise from the isoelectric point with a lowering of the pH until they reach a maximum; this maxi-

mum is, however, not identical. For P.D. it varies between 3.6 and 4.0, while for osmotic pressure it lies near 3.6.[7] With a further fall in pH both sets of curves show approximately the same steep drop.

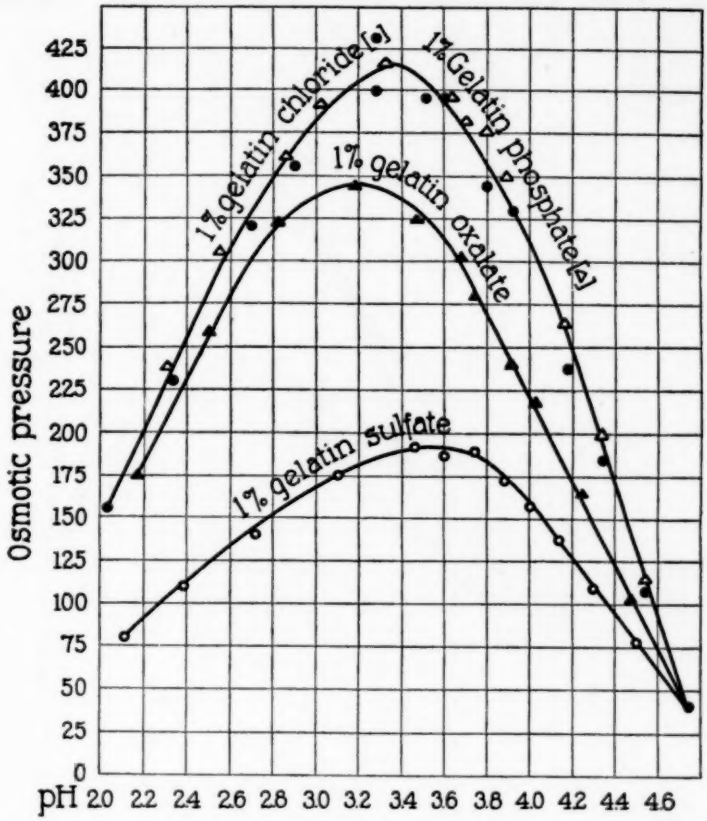


FIG. 5. Influence of pH and valency of anion on osmotic pressure of solutions of different gelatin-acid salts.

The second point of similarity is the influence of valency. The curves for P.D. (Fig. 6) are practically the same for gelatin chloride and gelatin phosphate, while the curve for P.D. is considerably lower in the case of gelatin sulfate.

[7] It may be stated incidentally that the maximum for the viscosity of gelatin solution also lies at a different pH, namely 3.0, from the maximum for osmotic pressure, which lies at pH 3.6.

These experiments offer an excellent opportunity to test our theory that the P.D. can be calculated with a fair degree of accuracy from the values of pH inside minus pH outside on the basis of Nernst's formula. Tables VIII, IX, and X show that this is true. The upper

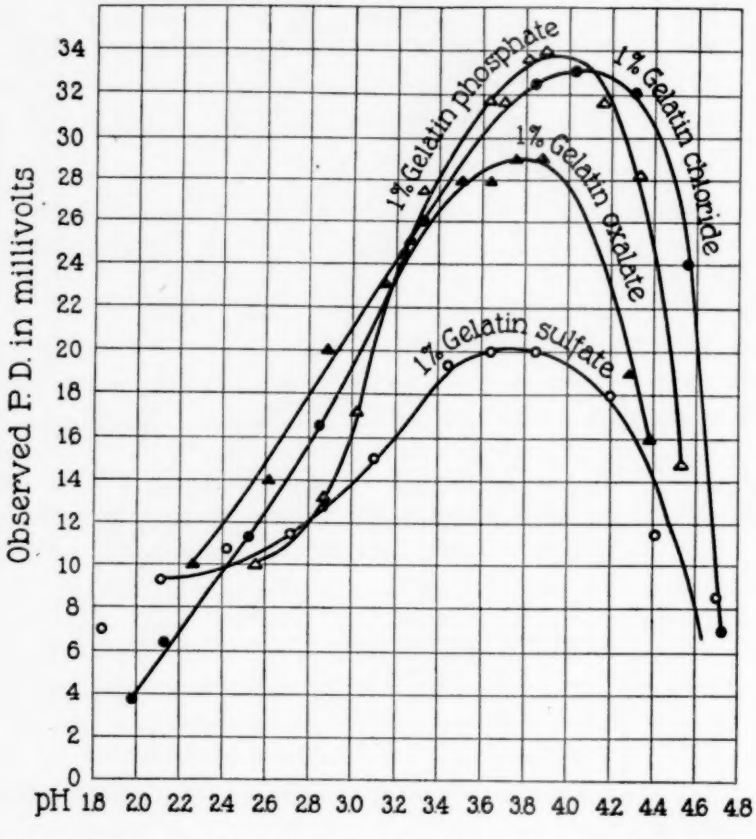


FIG. 6. Influence of pH and valency of anion on P.D. of solutions of different gelatin-acid salts. The curves in Fig. 6 are similar to (but not identical with) those in Fig. 5.

two horizontal rows give the pH inside and outside, the third horizontal row gives the difference pH inside minus pH outside, and the fourth row gives the P.D. calculated in millivolts by multiplying the values pH inside minus pH outside by 59. The last horizontal row

TABLE VIII.

*Influence of the Hydrogen Ion Concentration on pH Inside Minus pH Outside and on the P.D. of Gelatin Chloride Solutions af Equilibrium.*

| | Cc. 0.1 N HCl added to 100 cc. 1 per cent isoelectric gelatin. | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 4 | 6 | 8 | 10 | 12.5 | 15 | 20 | 30 | 40 | 50 |
| pH inside | 4.56 | 4.31 | 4.03 | 3.85 | 3.33 | 3.25 | 2.85 | 2.52 | 2.13 | 1.99 | 1.79 | 1.57 |
| pH outside | 4.14 | 3.78 | 3.44 | 3.26 | 2.87 | 2.81 | 2.53 | 2.28 | 2.00 | 1.89 | 1.72 | 1.53 |
| pH inside minus pH outside | 0.42 | 0.53 | 0.59 | 0.59 | 0.46 | 0.44 | 0.32 | 0.24 | 0.13 | 0.10 | 0.07 | 0.04 |
| P.D. calculated (millivolts) | +24.7 | +31.0 | +34.5 | +34.5 | +27.0 | +25.8 | +18.8 | +14.0 | +7.6 | +5.9 | +4.1 | +2.3 |
| P.D. observed (millivolts) | +24.0 | +32.0 | +33.0 | +32.5 | +26.0 | +24.5 | +16.5 | +11.2 | +6.4 | +4.8 | +3.7 | +2.1 |

TABLE IX.

*Influence of the Hydrogen Ion Concentration on pH Inside Minus pH Outside and on P.D. of Gelatin Phosphate Solutions at Equilibrium.*

| | Cc. M/10 $H_3PO_4$ added to 100 cc. 1 per cent isoelectric gelatin. | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 4 | 6 | 7 | 8 | 10 | 12.5 | 15 | 20 | 30 | 40 | 50 |
| pH inside | 4.79 | 4.54 | 4.31 | 3.98 | 3.68 | 3.56 | 3.38 | 3.24 | 3.02 | 2.67 | 2.42 | 2.12 | 1.92 | 1.74 |
| pH outside | 4.70 | 4.10 | 3.77 | 3.40 | 3.14 | 3.04 | 2.90 | 2.80 | 2.66 | 2.39 | 2.22 | 1.98 | 1.83 | 1.67 |
| pH inside minus pH outside | 0.09 | 0.44 | 0.54 | 0.58 | 0.54 | 0.52 | 0.48 | 0.44 | 0.36 | 0.28 | 0.20 | 0.14 | 0.09 | 0.07 |
| P.D. calculated (millivolts) | +5.3 | +25.8 | +31.7 | +34.0 | +31.7 | +30.5 | +28.0 | +25.8 | +21.2 | +16.4 | +11.7 | +8.2 | +5.3 | +4.1 |
| P.D. observed (millivolts) | +5.7 | +27.0 | +29.0 | +30.0 | +30.6 | +29.6 | +26.5 | +24.4 | +22.3 | +17.7 | +15.6 | +11.4 | +9.9 | +7.3 |

TABLE X.

*Influence of the Hydrogen Ion Concentration on pH Inside Minus pH Outside and on P.D. of Gelatin Sulfate Solutions at Equilibrium.*

| | Cc. 0.1 N $H_2SO_4$ added to 100 cc. 1 per cent isoelectric gelatin. | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 4 | 6 | 7 | 8 | 10 | 12.5 | 15 | 20 | 30 | 40 | 50 |
| pH inside | 4.76 | 4.52 | 4.34 | 3.98 | 3.73 | 3.49 | 3.41 | 3.12 | 2.78 | 2.47 | 2.16 | 2.06 | 1.84 | 1.57 |
| pH outside | 4.61 | 4.20 | 3.99 | 3.60 | 3.38 | 3.18 | 3.14 | 2.88 | 2.61 | 2.35 | 2.09 | 2.00 | 1.80 | 1.54 |
| pH inside minus pH outside | 0.15 | 0.32 | 0.35 | 0.38 | 0.35 | 0.31 | 0.27 | 0.24 | 0.17 | 0.12 | 0.07 | 0.06 | 0.04 | 0.03 |
| P.D. calculated (millivolts) | +8.8 | +18.8 | +20.5 | +22.2 | +20.5 | +18.1 | +15.8 | +14.0 | +10.0 | +7.0 | +4.1 | +3.5 | +2.4 | +1.8 |
| P.D. observed (millivolts) | +6.3 | +16.3 | +18.4 | +19.0 | +19.0 | +17.4 | +15.8 | +13.7 | +10.5 | +8.4 | +7.4 | +5.8 | +4.7 | +3.7 |

gives the observed P.D. in millivolts. The agreement between observed and calculated P.D. is sufficiently close.

### *VI. Hydrogen Ion and Chlorine Ion Potentials.*

The equation for the equilibrium condition between gelatin chloride solution and water is as stated above,

$$x^2 = y\,(y + z)$$

where $x$ is the concentration of H and Cl ions in the outside solution, $y$ the concentration of the H and Cl ions of the free HCl inside the gelatin chloride solution, and $z$ the concentration of the Cl ions held by the gelatin.

If we write this equation in the form

$$\frac{y}{x} = \frac{x}{y + z}$$

$\frac{y}{x}$ is the ratio of hydrogen ion concentration inside over the hydrogen ion concentration outside; and $\frac{x}{y + z}$ is the ratio of the concentration of the chlorine solution outside over the chlorine solution inside. Since

$$\log \frac{y}{x} = \text{pH inside minus pH outside}$$

and

$$\log \frac{x}{y + z} = \text{pCl outside minus pCl inside}$$

it follows that

$$\text{pH inside minus pH outside} = \text{pCl outside minus pCl inside}$$

It was easy to put this consequence of Donnan's theory to a test and some of the experiments described in the preceding chapter were selected for this purpose. Inside the collodion bag was a 1 per cent solution of gelatin chloride of different pH, outside water. After 18 hours equilibrium was established between inside and outside solutions and the pH as well as the pCl were ascertained. The pCl

was determined in two different ways in the two experiments; in one experiment it was determined with the potentiometer, in the other it was determined in the gelatin chloride solution by titration with NaOH according to the method described in a preceding paper.[8] Both methods of determining the pCl led to the result that the values pCl outside minus pCl inside were for the same solution at the point of equilibrium equal to the value pH inside minus pH outside (within the limits of accuracy of the experiments). The pCl outside was identical with the pH outside, since the outside solution contained only free HCl. The values of pH were all determined potentiometrically.

TABLE XI.

Experiment 1. pCl determined by titration.

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| pH of gelatin chloride solution at equilibrium | 4.13 | 3.69 | 3.30 | 3.10 | 2.92 | 2.78 | 2.46 | 2.26 | 2.01 | 1.76 |
| pH inside minus pH outside | 0.56 | 0.58 | 0.50 | 0.49 | 0.44 | 0.44 | 0.33 | 0.23 | 0.15 | 0.10 |
| pCl outside minus pCl inside | 0.48 | 0.51 | 0.59 | 0.44 | 0.44 | 0.38 | 0.35 | 0.22 | 0.15 | 0.11 |

Experiment 2. pCl determined electrometrically.

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| pH of gelatin chloride solution at equilibrium | 4.04 | 3.92 | 3.78 | 3.61 | 3.46 | 3.16 | 2.73 | 2.36 | 2.04 | 1.73 |
| pH inside minus pH outside | 0.60 | 0.62 | 0.66 | 0.55 | 0.50 | 0.43 | 0.30 | 0.20 | 0.12 | 0.07 |
| pCl outside minus pCl inside | 0.55 | 0.60 | 0.57 | 0.50 | 0.53 | 0.38 | 0.32 | 0.17 | 0.12 | 0.07 |

Nernst's formula leads therefore to the same theoretical P.D. regardless of the fact whether we calculate the P.D. on the basis of the difference pH inside minus pH outside or pCl outside minus pCl inside. It is also obvious that both assumptions lead to the same sign of charge of the gelatin chloride solution. If we assume that the P.D. is determined by differences in the hydrogen ion concentration the outside solution is concentrated and the inside solution dilute; if the P.D. is determined by differences in the concentration of the Cl ions the inside solution is concentrated and the outside solution dilute. Since the common ion is positive in the former and negative in the latter case, the gelatin solution becomes positive in both cases.

[8] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 559.

| outside solution. | | inside solution. |
|---|---|---|
| $-$ $H^+$ concentrated<br>$-$ $Cl^-$ dilute | membrane | $H^+$ dilute $+$<br>$Cl^-$ concentrated $+$ |

The facts of this last chapter prove beyond doubt that the equation $x^2 = y\,(y + z)$ is the correct expression for the Donnan membrane equilibrium between acid-salts of proteins with monovalent anion and water. The close analogy between the variation of the membrane potentials and the other osmotic properties of solutions of protein-acid salts, such as osmotic pressure, viscosity, and swelling, suggests that an attempt be made to derive the variations of these latter properties directly from the equation for the Donnan equilibrium. This has already been done by Procter for the swelling of gelatin chloride.

## SUMMARY.

1. It is shown that a neutral salt depresses the potential difference which exists at the point of equilibrium between a gelatin chloride solution contained in a collodion bag and an outside aqueous solution (without gelatin). The depressing effect of a neutral salt on the P.D. is similar to the depression of the osmotic pressure of the gelatin chloride solution by the same salt.

2. It is shown that this depression of the P.D. by the salt can be calculated with a fair degree of accuracy on the basis of Nernst's logarithmic formula on the assumption that the P.D. which exists at the point of equilibrium is due to the difference of the hydrogen ion concentration on the opposite sides of the membrane.

3. Since this difference of hydrogen ion concentration on both sides of the membrane is due to Donnan's membrane equilibrium this latter equilibrium must be the cause of the P.D.

4. A definite P.D. exists also between a solid block of gelatin chloride and the surrounding aqueous solution at the point of equilibrium and this P.D. is depressed in a similar way as the swelling of the gelatin chloride by the addition of neutral salts. It is shown that the P.D. can be calculated from the difference in the hydrogen ion concentration inside and outside the block of gelatin at equilibrium.

5. The influence of the hydrogen ion concentration on the P.D. of a gelatin chloride solution is similar to that of the hydrogen ion concentration on the osmotic pressure, swelling, and viscosity of gelatin solutions, and the same is true for the influence of the valency of the anion with which the gelatin is in combination. It is shown that in all these cases the P.D. which exists at equilibrium can be calculated with a fair degree of accuracy from the difference of the pH inside and outside the gelatin solution on the basis of Nernst's logarithmic formula by assuming that the difference in the concentration of hydrogen ions on both sides of the membrane determines the P.D.

6. The P.D. which exists at the boundary of a gelatin chloride solution and water at the point of equilibrium can also be calculated with a fair degree of accuracy by Nernst's logarithmic formula from the value pCl outside minus pCl inside. This proves that the equation $x^2 = y\,(\,y + z)$ is the correct expression for the Donnan membrane equilibrium when solutions of protein-acid salts with monovalent anion are separated by a collodion membrane from water. In this equation $x$ is the concentration of the H ion (and the monovalent anion) in the water, $y$ the concentration of the H ion and the monovalent anion of the free acid in the gelatin solution, and $z$ the concentration of the anion in combination with the protein.

7. The similarity between the variation of P.D. and the variation of the osmotic pressure, swelling, and viscosity of gelatin, and the fact that the Donnan equilibrium determines the variation in P.D. raise the question whether or not the variations of the osmotic pressure, swelling, and viscosity are also determined by the Donnan equilibrium.

The measurements referred to in this paper were made by the writer's technical assistants, Mr. M. Kunitz and Mr. N. Wuest, to whom the writer wishes to express his indebtedness.

# DONNAN EQUILIBRIUM AND THE PHYSICAL PROPERTIES OF PROTEINS.

## II. Osmotic Pressure.

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### I. INTRODUCTION.

It was pointed out in two preceding papers[1] that the curves representing the influence of electrolytes on the membrane potentials between gelatin solutions and water at the point of equilibrium show a marked similarity to the curves representing the influence of the same electrolytes on osmotic pressure, swelling, and viscosity of gelatin. There was no doubt left that the Donnan equilibrium accounted quantitatively for these variations in the case of membrane potentials[2] and the question therefore arose whether the same equilibrium condition can account also for the corresponding variations of the osmotic pressure of protein solutions. An attempt was, therefore, made to calculate the osmotic pressures on the basis of the Donnan equilibrium in order to find out whether the curves for the calculated values would show the characteristics of the curves representing the observed values for osmotic pressures. The depressing effect of the addition of a neutral salt on the osmotic pressure of colloidal solutions was predicted by Donnan and offered no theoretical difficulty.[3] The real test was whether the Donnan equilibrium would be able to account for the peculiar curves obtained when the observed osmotic pressures of 1 per cent solutions of gelatin-acid salts are plotted as a function of the pH.

[1] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 557, 667.

[2] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 667.

[3] Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572. Donnan, F. G., and Harris, A. B., *J. Chem. Soc.*, 1911, xcix, 1554. Donnan, F. G., and Garner, W. E., *J. Chem. Soc.*, 1919, cxv, 1313.

Fig. 1 shows a set of such curves where the abscissæ are the pH of the gelatin solution at equilibrium, and the ordinates the observed osmotic pressures. There are two outstanding peculiarities in these curves, namely, first, that they all rise from a minimum at pH 4.7 (the isoelectric point of gelatin) until they reach a maximum at pH

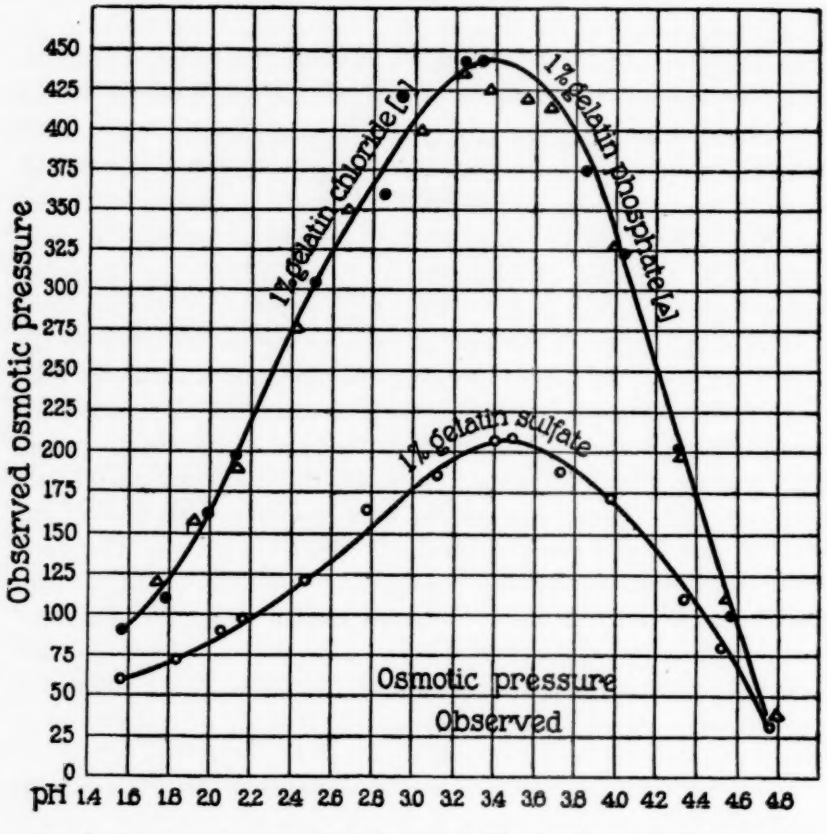


FIG. 1. Curves representing the influence of pH and valency of anion on osmotic pressure of solutions of gelatin-acid salts containing 1 gm. of originally isoelectric gelatin in 100 cc. solution. The curves for gelatin chloride and gelatin phosphate are identical since the anions, Cl and $H_2PO_4$, of these two gelatin salts are monovalent. The curve for gelatin sulfate is less than half as high as the curve for the two other salts because the anion of gelatin sulfate is bivalent. Both curves rise from the isoelectric point at 4.7 to a maximum at pH about 3.4 or 3.5, and then drop rapidly again.

about 3.5, and then drop again; and second, that only the valency of the ion in combination with a protein influences its osmotic pressure (or degree of swelling, etc.), while the specific nature of the ion aside from its valency has no influence. The latter fact is really the crucial point which decides between colloid chemistry and classical physical chemistry. If only the valency of the ion in combination with a protein is of importance and if gelatin-acid salts of the same pH and the same concentration of originally isoelectric gelatin have the same osmotic pressure, provided the anion of the gelatin-acid salt has the same valency, a suspicion must arise that we are dealing with some equilibrium condition for which classical physical chemistry is able to account. The writer has shown in preceding papers that gelatin chloride, nitrate, acetate, succinate, tartrate, citrate, and phosphate have at the same pH and the same concentration of originally isoelectric gelatin the same osmotic pressure; and it was shown by titration curves that the anion in all these salts is monovalent. The titration curves show also that the anion in gelatin sulfate is bivalent and we have found that the osmotic pressure of gelatin sulfate is less than half that of gelatin chloride or phosphate at the same pH and for the same concentration of originally isoelectric gelatin.[4]

Fig. 1 illustrates this valency effect in the observed osmotic pressure. The curves for the observed osmotic pressure of gelatin chloride and gelatin phosphate are identical while the curve for gelatin sulfate is considerably lower.

It is the purpose of this paper to show that we can calculate with a fair degree of accuracy the osmotic pressure of gelatin solutions on the assumption of the validity of Donnan's equilibrium equation and the validity of van't Hoff's theory of osmotic pressure.

## *II. Theoretical Data.*

A gelatin chloride solution contains free hydrochloric acid, gelatin chloride (which dissociates electrolytically like any other salt in watery solution), and non-ionogenic protein molecules. A 1 per cent gelatin chloride solution of about pH 3.5 is in equilibrium with a

[4] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 85, 247, 391; *Science*, 1920, lii, 449.

HCl solution (free from protein) of a pH of about 3.0, both solutions being separated by a collodion membrane.

Let $y$ be the concentration of the H and Cl ions of the free HCl inside a gelatin chloride solution (containing 1 gm. of originally isoelectric gelatin in 100 cc.), $z$ the concentration of the Cl ions held by the gelatin ions, and $a$ the sum of the gelatin ions and non-ionized molecules of gelatin. For the sake of simplification we assume complete electrolytic dissociation of the gelatin chloride and of the HCl. In this case the real osmotic pressure of the inside solution is determined by

$$2y + z + a$$

Since, however, the outside solution is at equilibrium not $H_2O$ but a HCl solution—in the example selected of about pH 3.0—the *observed* osmotic pressure is the difference between the osmotic pressure of the inside solution against $H_2O$ and the osmotic pressure of the outside solution.

Let $x$ be the concentration of the H ions in the outside solution, then the osmotic pressure of the outside solution is determined by $2x$.

Hence the observed osmotic pressure $P_o$ of the gelatin chloride solution is determined by

$$P_o = 2y + z + a - 2x$$

$P_o$ is observed experimentally, $y$ can be calculated from the pH inside, and $x$ from the pH outside.

$z$ can be calculated from Donnan's equilibrium equation in the form given it by Procter[5]

$$x^2 = y\,(y + z) \qquad (1)$$

$$z = \frac{(x + y)\ (x - y)}{y}.$$

where $x$, $y$, and $z$ have the significance stated above. We have seen in the preceding paper that this equation leads to correct results in regard to the P.D.

[5] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

$a$ is unknown, and we therefore can only calculate for the present the values of

$$2y + z - 2x$$

If we express the theoretical osmotic pressure of a grammolecular solution in terms of mm. pressure of a column of $H_2O$ we get (with correction for a temperature of 24°C.)

$$22.4 \times 760 \times 13.6 \times \frac{297}{273} = 2.5 \times 10^5$$

In other words, a theoretical pressure of 2.5 mm. $H_2O$ corresponds to a concentration of $10^{-5}$ N. In the following tables all concentrations are expressed in terms of $10^{-5}$ N and hence we only need to multiply the values for $2y + z - 2x$ given in our tables by 2.5 to obtain the calculated osmotic pressure of the gelatin solution (neglecting the osmotic pressure of the gelatin ions and molecules).

Equation (1) holds in the case of solutions of all gelatin-acid salts with monovalent anion; *i.e.* gelatin chloride, acetate, phosphate, tartrate, citrate, etc. When, however, the anion of a gelatin-acid salt is divalent, as in the case of gelatin sulfate, the equilibrium equation becomes one of the third degree. If $x$ be the hydrogen ion concentration of the outside solution, the concentration of the $SO_4$ ion in the outside solution becomes $\frac{x}{2}$. If $y$ be the concentration of the H ions of the free sulfuric acid in the inside solution, $\frac{y}{2}$ is the concentration of the $SO_4$ ions of the free acid inside the gelatin sulfate solution. In the case of gelatin chloride $z$ represented the concentration of chlorine ions in combination with the gelatin; hence $\frac{z}{2}$ will represent the concentration of $SO_4$ ions in combination with the same number of gelatin ions.

The equilibrium equation, therefore, assumes in the case of gelatin sulfate the following form

$$x^2 \cdot \frac{x}{2} = y^2 \frac{(y + z)}{2} \qquad (2)$$

From equation (2) follows

$$z = \frac{x^3 - y^3}{y^2}$$

The osmotic pressure of the gelatin solution should therefore be calculated from the following values (omitting the share of the osmotic pressure due to the gelatin)

$$\frac{3}{2}y + \frac{z}{2} - \frac{3}{2}x$$

### *III. The Calculated Curves.*

Solutions containing 1 gm. of originally isoelectric gelatin in 100 cc. and containing different quantities of acid were prepared. Collodion bags cast in the form of Erlenmeyer flasks of 50 cc. volume were filled with the 1 per cent solution of a gelatin-acid salt and put into a beaker containing 350 cc. of $H_2O$. In order to accelerate the establishment of the equilibrium between inside and outside solutions a certain amount of acid was added to the outside water (*e.g.*, HCl in the experiments with gelatin chloride, $H_3PO_4$ in the experiments with gelatin phosphate, etc.). Each Erlenmeyer flask was closed with a rubber stopper perforated by a glass tube serving as a manometer. All this was described in more detail in previous publications.

In Fig. 2 are plotted the values of the calculated osmotic pressures for 1 per cent solutions of gelatin chloride, gelatin phosphate, and gelatin sulfate, and Tables I, II, and III give the data on the basis of which the curves in Fig. 2 are calculated. The experiments from which these calculations were made are identical with the experiments from which the curves for the observed osmotic pressures in Fig. 1 were plotted. The abscissæ in Fig. 2 are the pH in the inside solution at the point of equilibrium, the ordinates are the values for osmotic pressure calculated on the basis of the Donnan equilibrium as discussed before. The reader will notice that the three curves plotted in Fig. 2 show not only the same qualitative characteristics as the curves for the observed osmotic pressures in Fig. 1, but show them almost quantitatively; except that a correction for the value of osmotic pressure due to the gelatin particles itself may have to be

TABLE I.

*Gelatin Chloride.*

*Observed and Calculated Osmotic Pressures of Gelatin Chloride Containing 1 gm. of Originally Isoelectric Gelatin in 100 cc. Solution at Equilibrium.*

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pH inside | 4.56 | 4.31 | 4.03 | 3.85 | 3.33 | 3.25 | 2.85 | 2.52 | 2.13 | 1.99 | 1.79 | 1.57 |
| pH outside | 4.14 | 3.78 | 3.44 | 3.26 | 2.87 | 2.81 | 2.53 | 2.28 | 2.00 | 1.89 | 1.72 | 1.53 |
| $y = C_H$ inside $\times 10^5$ | 2.7 | 4.9 | 9.3 | 14.1 | 46.8 | 56.2 | 141 | 302 | 741 | 1023 | 1622 | 2692 |
| $x = C_H$ outside $\times 10^5$ | 7.2 | 16.6 | 36.3 | 54.9 | 135.0 | 155.0 | 295 | 524 | 1000 | 1288 | 1905 | 2951 |
| $z = \frac{(x+y)(x-y)}{y}$ | 16.5 | 51.4 | 132.5 | 200.0 | 343.0 | 372.0 | 477 | 608 | 609 | 600 | 612 | 544 |
| $2y + z - 2x$ | 7.5 | 28.0 | 78.5 | 118.4 | 166.6 | 174.4 | 169 | 164 | 91 | 70 | 46 | 26 |
| Observed osmotic pressure | 100 | 202 | 322 | 375 | 443 | 442 | 360 | 303 | 198 | 162 | 110 | 90 |
| Calculated osmotic pressure, neglecting osmotic pressure of protein | 19 | 70 | 196 | 296 | 416 | 436 | 422 | 410 | 227 | 175 | 115 | 65 |

TABLE II.

*1 Per Cent Gelatin Phosphate.*

*Observed and Calculated Osmotic Pressures at Equilibrium.*

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pH inside | 4.79 | 4.54 | 4.31 | 3.98 | 3.68 | 3.56 | 3.38 | 3.24 | 3.02 | 2.67 | 2.42 | 2.12 | 1.92 | 1.74 |
| pH outside | 4.70 | 4.10 | 3.77 | 3.40 | 3.14 | 3.04 | 2.90 | 2.80 | 2.66 | 2.39 | 2.22 | 1.98 | 1.83 | 1.67 |
| $y = C_H$ inside $\times 10^5$ | 1.6 | 2.9 | 4.9 | 10.5 | 20.9 | 27.5 | 41.7 | 57.5 | 95.5 | 213.8 | 380.2 | 758.6 | 1202 | 1820 |
| $x = C_H$ outside $\times 10^5$ | 2.0 | 7.9 | 16.9 | 39.8 | 72.4 | 91.2 | 125.9 | 158.5 | 218.8 | 407.4 | 602.6 | 1047.0 | 1479 | 2138 |
| $z = \frac{(x+y)(x-y)}{y}$ | 0.9 | 18.6 | 53.3 | 140.0 | 228.0 | 231.0 | 338.0 | 380.0 | 405.0 | 556.0 | 575.0 | 686.0 | 617 | 690 |
| $2y + z - 2x$ | 0.1 | 8.6 | 31.3 | 81.4 | 125.0 | 103.6 | 169.6 | 178.0 | 158.0 | 169.0 | 130.0 | 109.0 | 63 | 54 |
| Observed osmotic pressure | 34 | 111 | 199 | 328 | 416 | 420 | 426 | 436 | 401 | 350 | 275 | 190 | 158 | 121 |
| Calculated osmotic pressure, neglecting osmotic pressure of protein | | 22 | 77 | 203 | 310 | 258 | 423 | 445 | 395 | 420 | 324 | 273 | 157 | 135 |

TABLE III.

*1 Per Cent Gelatin Sulfate.*

*Observed and Calculated Osmotic Pressure at Equilibrium.*

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pH inside | 4.76 | 4.52 | 4.34 | 3.98 | 3.73 | 3.49 | 3.41 | 3.12 | 2.78 | 2.47 | 2.16 | 2.06 | 1.84 | 1.57 |
| pH outside | 4.61 | 4.20 | 3.99 | 3.60 | 3.38 | 3.18 | 3.14 | 2.88 | 2.61 | 2.35 | 2.09 | 2.00 | 1.80 | 1.54 |
| $y = C_H$ inside $\times 10^5$ | 1.7 | 3.0 | 4.6 | 10.4 | 18.6 | 32.3 | 38.9 | 75.9 | 166 | 339 | 692 | 871 | 1445 | 2692 |
| $x = C_H$ outside $\times 10^5$ | 3.1 | 6.3 | 10.2 | 25.1 | 41.7 | 66.0 | 72.4 | 131.8 | 245.5 | 447 | 813 | 1000 | 1585 | 2884 |
| $z = \frac{x^3 - y^3}{y^2}$ | 8.3 | 24.7 | 45.8 | 136 | 191.5 | 243 | 212 | 322 | 390 | 435 | 433 | 449 | 466 | 620 |
| $\frac{3}{2}y + \frac{z}{2} - \frac{3}{2}x$ | 2 | 7.35 | 14.5 | 46 | 64 | 71 | 55.8 | 77 | 77 | 55 | 37 | 31 | 23 | 20 |
| Observed osmotic pressure | 33 | 79 | 110 | 172 | 188 | 208 | 208 | 185 | 164 | 122 | 98 | 89 | 72 | 61 |
| Calculated osmotic pressure, neglecting osmotic pressure of protein | 5 | 18.5 | 36 | 115 | 160 | 178 | | 192 | 192 | 138 | 92.5 | 77.5 | 57.5 | 50 |

added, a point which will be discussed in the next chapter. What is of importance here is the following. The curves for osmotic pressure calculated on the basis of the Donnan equilibrium and plotted

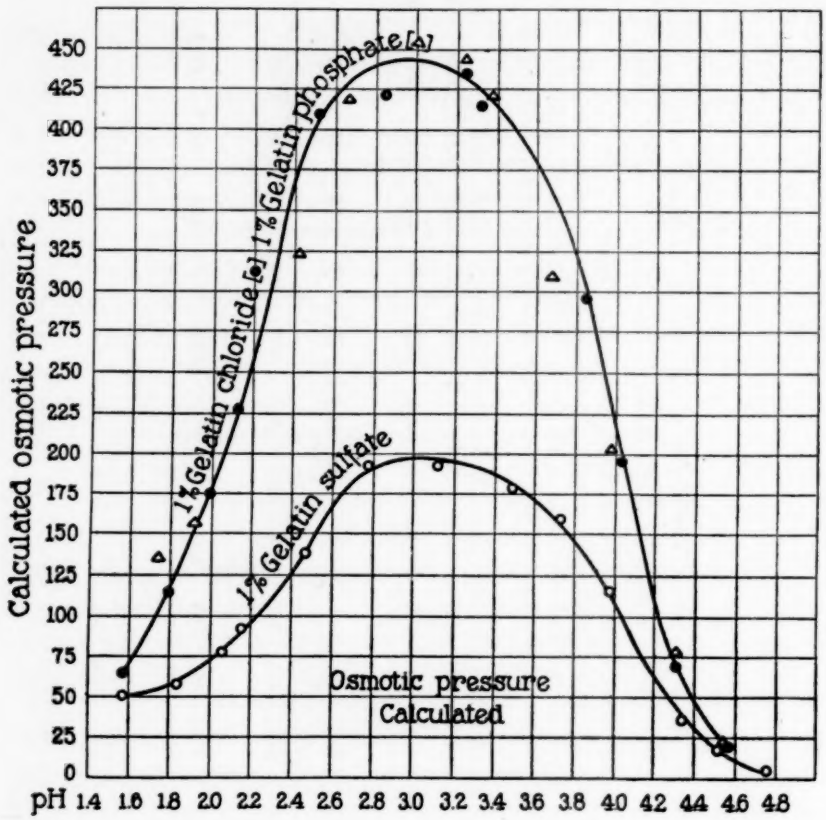


FIG. 2. Calculated curves of osmotic pressure taken from the data of the experiments represented in Fig. 1. The calculation is made on the basis of the validity of Donnan's theory of membrane equilibrium. The calculations lead to curves resembling the curves in Fig. 1 in all essential points, in regard to valency effect of the anion, as well as in regard to influence of pH. (See legend in Fig. 1.)

in Fig. 2 resemble the curves for the osmotic pressure observed in the same experiments represented in Fig. 1 in the following essential points.

1. The curve for the calculated osmotic pressure of gelatin chloride is identical with the curve for the calculated osmotic pressure of gelatin phosphate, and the same is true for the two corresponding curves representing the observed osmotic pressures (Figs. 1 and 2).

2. The curve for the calculated osmotic pressure of gelatin sulfate is a little less than half as high as the curves for the calculated osmotic pressures of gelatin chloride and gelatin phosphate; and the same is true for the curves representing the observed osmotic pressures of gelatin sulfate and gelatin chloride.

3. All the curves in Figs. 1 and 2 rise from a minimum at pH 4.7, reach a maximum (which lies at pH 3.4 or 3.5 for the observed, and at 3.0 for the calculated curves), and then drop again as steeply as they rose on the other side. Moreover, the absolute values of observed and calculated osmotic pressures agree almost quantitatively, a fact which will be discussed more fully in Chapter V.

It may be added that the curve for the calculated values of the osmotic pressure of gelatin oxalate solutions agrees also with the curve for the observed values of the osmotic pressure of solutions of the same gelatin salt, both being slightly lower than the curves for gelatin chloride.

We can therefore say that (with the exception of two minor discrepancies to be discussed further on) the Donnan equilibrium accounts not only qualitatively but almost quantitatively for (*a*) *the valency effect of the anion with which the gelatin is in combination;* (*b*) *for the effect of the pH*.

Thus two of the most puzzling problems of the colloid chemistry of proteins seem to find their solution on the basis of classical physical chemistry.

### *IV. The Presumable Osmotic Pressure of Gelatin Particles.*

The question now arises what the possible share of the protein particles in the osmotic pressure may be. Different concentrations of gelatin phosphate from 2 per cent to ½ per cent were prepared, all having a pH of 3.5. The gelatin phosphate solutions were put into Erlenmeyer flasks of 50 cc. volume, connected with a glass tube serving as a manometer as described, and these flasks were put into beakers containing 350 cc. of $H_2O$, the pH of which was brought at

the beginning of the experiment to 3.5 through the addition of $H_3PO_4$. When the bags containing gelatin phosphate solutions are put into water the latter diffuses rapidly into the gelatin solution thereby lowering the concentration of the gelatin solution. To avoid this error so much gelatin phosphate solution was poured into each bag and glass tube that at the beginning of the experiment the liquid reached already to about that level which from preceding experiments we knew the gelatin solution would reach in the manometer at the point of osmotic equilibrium. All experiments were made in duplicate. In addition to the osmotic pressure we measured the pH inside and outside after equilibrium was reached. From these latter data the osmotic pressure due to the H and $PO_4$ ions could be calculated, being equal to

$$(2y + z - 2x) \times 2.5 \text{ mm } H_2O$$

By deducting this value from the observed osmotic pressure in each case it was hoped to obtain a rational value for the share of the protein particles in the observed osmotic pressure. Table IV gives the results.

The reader's attention is called to the last two rows of figures (Table IV) giving the difference between the observed and the calculated osmotic pressures, since if this difference actually represents the osmotic pressure due to the gelatin particles, the figures should be in direct proportion to the concentration of the gelatin. The experiments were all made in duplicate to give some idea of the magnitude of error and it is obvious that the error may be considerable, 25 per cent or more, because the errors in the observed and the calculated values are additive. Thus the "difference" is for $\frac{3}{4}$ per cent solution in one case 92, in the other 61, a variation of 50 per cent! If we take this into consideration we may conclude that the differences between the observed and the calculated osmotic pressures are compatible with the idea that the difference is the value for the osmotic pressure due to the gelatin particles in solution.

This would lead us to the conclusion that the osmotic pressure due to the gelatin particles in a 1 per cent solution (of originally isoelectric gelatin) of gelatin phosphate of pH 3.60 is about 100 mm. $H_2O$. Since the osmotic pressure of 1 grammolecule is about 250,000 mm.

TABLE IV.

*Influence of Concentration of Gelatin Phosphate of pH of About 3.6 on the Osmotic Pressure. (All Experiments Were Made in Two Sets.)*

| | Concentration of gelatin in per cent. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 2 | 1½ | 1½ | 1 | 1 | ¾ | ¾ | ½ | ½ |
| pH inside at equilibrium | 3.64 | 3.66 | 3.60 | 3.60 | 3.65 | 3.66 | 3.60 | 3.60 | 3.61 | 3.62 |
| pH outside at equilibrium | 3.02 | 3.02 | 3.02 | 3.01 | 3.12 | 3.11 | 3.14 | 3.12 | 3.21 | 3.19 |
| $y = C_H$ inside $\times 10^5$ | 22.9 | 21.9 | 25.1 | 25.1 | 22.4 | 21.9 | 25.1 | 25.1 | 24.6 | 24.0 |
| $x = C_H$ outside $\times 10^5$ | 95.5 | 95.5 | 95.5 | 97.7 | 75.9 | 77.6 | 72.4 | 75.9 | 61.7 | 64.6 |
| $z = \frac{(x+y)(x-y)}{y}$ | 375 | 395 | 338 | 355 | 235 | 253 | 184 | 204 | 130 | 150 |
| $2y + z - 2x$ | 230 | 248 | 197 | 210 | 128 | 142 | 89 | 102 | 56 | 69 |
| Observed osmotic pressure | 860 | 860 | 715 | 680 | 420 | 445 | 314 | 316 | 186 | 186 |
| Calculated osmotic pressure (ignoring gelatin) | 576 | 620 | 493 | 523 | 320 | 355 | 222 | 255 | 140 | 172 |
| Difference (osmotic pressure due to gelatin) | 284 | 240 | 222 | 157 | 100 | 90 | 92 | 61 | 46 | 14 |
| Mean | 262 | | 190 | | 95 | | 73 | | 26 | |

$H_2O$ and since 1 liter of a 1 per cent solution of gelatin contains 10 gms. of gelatin, the molecular weight of gelatin should be expected to be in the neighborhood of 25,000. The experiment just described for gelatin phosphate was repeated for gelatin chloride, with similar results.

According to Dakin's[6] recent analyses gelatin contains 1.4 per cent phenylalanine. Since 1 molecule of gelatin cannot contain less than 1 molecule of phenylalanine and since the molecular weight of this

TABLE V.

*Influence of Concentration of Albumin Chloride of pH of About 3.4 on the Osmotic Pressure.*

| | Concentration of Egg Albumin in per cent. | | | | | |
|---|---|---|---|---|---|---|
| | 4 | 3 | 2 | 1 | ½ | ¼ |
| pH inside at equilibrium..... | 3.34 | 3.32 | 3.38 | 3.40 | 3.40 | 3.40 |
| pH outside at equilibrium.... | 2.98 | 2.97 | 3.07 | 3.14 | 3.19 | 3.24 |
| $y = C_H$ inside $\times 10^5$..... | 45.7 | 47.9 | 41.7 | 39.8 | 39.8 | 39.8 |
| $x = C_H$ outside $\times 10^5$..... | 104.7 | 107.2 | 85.1 | 72.4 | 64.5 | 57.5 |
| $z = \frac{(x+y)(x-y)}{y}$..... | 194.0 | 192.0 | 132.0 | 92.0 | 64.6 | 43.3 |
| $2y + z - 2x$..... | 76 | 74 | 45 | 27 | 15 | 8 |
| Observed osmotic pressure.... | 776 | $555 + x$ | 375 | 163 | 75 | 36 |
| Calculated osmotic pressure (ignoring albumin)..... | 190 | 185 | 113 | 67 | 39 | 20 |
| Difference (osmotic pressure due to albumin)..... | 586 | $370 + x$ | 262 | 96 | 36 | 16 |

amino-acid is 165 the lowest possible molecular weight of gelatin is 11,800. If a molecule of gelatin contains two molecules of phenylalanine, the molecular weight should be about 23,600. This would be approximately the figure we might expect from the data of Table IV on the assumption that the differences in the last two rows may be considered to be the values of the osmotic pressure of the protein particles.

[6] Dakin, H. D., *J. Biol. Chem.*, 1920, xliv, 499.

A similar experiment was made with different concentrations of solutions of the chloride of crystalline egg albumin. The original pH of the albumin chloride solution was 3.5 and that of the outside solution 3.0. After equilibrium was established the pH both inside and outside was slightly changed as is shown in Table V. The osmotic pressures for $\frac{1}{4}$ to 4 per cent solutions of albumin chloride were measured and calculated for $2y + z - 2x$. The difference, which should be the osmotic pressure of the albumin particles in solution, is found in the last row. It is almost identical with the difference found for gelatin chloride for the same concentration of gelatin.

*V. Difference between the Curves for Calculated and Observed Values.*

The curves representing the values for calculated osmotic pressures differ in one or two respects from the curves representing the values for the observed osmotic pressures. These differences are not great but they are constant and can therefore not be due to an accidental error. Fig. 3 shows the difference between the curves for the observed and the calculated osmotic pressures in the case of a gelatin chloride solution containing 1 gm. of originally isoelectric gelatin in 100 cc. If we start with the ascending branch of the two curves of Fig. 3, we notice that the observed osmotic pressures for pH 4.4 to pH 3.8 are about 100 mm. higher in each case than the calculated pressures. It may be a mere accident but 100 mm. happens to be the approximate value for the osmotic pressure of 1 gm. of gelatin in 100 cc. and if the gelatin particles participate in the osmotic pressure of solutions of gelatin salts our calculated values should be about 100 mm. lower than the observed values for the same pH in the case of a 1 per cent gelatin solution. Near the summit the difference becomes a little less but we have seen that we may expect such irregularities on account of experimental error. Besides at pH 3.4 the concentration of the gelatin solution was diminished by about 20 per cent on account of diffusion of water into the gelatin bag. The difference in the ascending branch of the observed and calculated values occurs in every experiment.

The second constant difference between the curves for observed and calculated osmotic pressures lies in the fact that the drop in the calculated curves begins at a lower pH than the drop for the curves of

observed values. This or both discrepancies may be due to a constant experimental error or they may find their explanation in the influence of one or more factors not taken into consideration in our calculations.

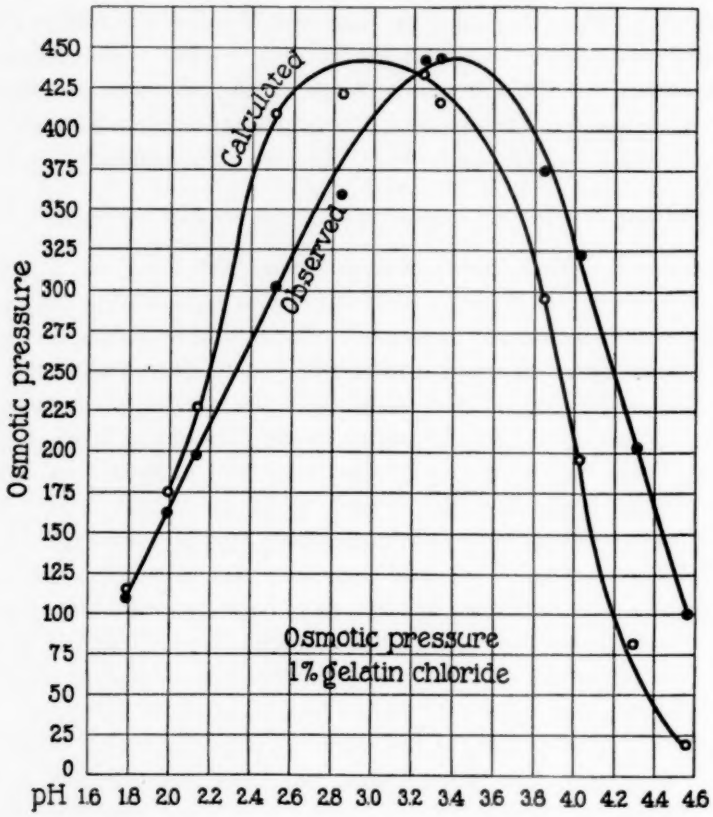


FIG. 3. Showing agreement and minor discrepancies between the curves of observed and calculated osmotic pressures of 1 per cent gelatin chloride solutions.

The calculated and observed curves for gelatin phosphate are identical with those for gelatin chloride given in Fig. 3, and we therefore omit these curves.

Fig. 4 gives the curves for observed and calculated values for gelatin sulfate. It seemed of interest to calculate the osmotic pres-

sures from some of our older experiments on the osmotic pressure of crystalline egg albumin (containing 1 gm. of isoelectric albumin in 100 cc. solution). Fig. 5 gives a comparison of the curves for the

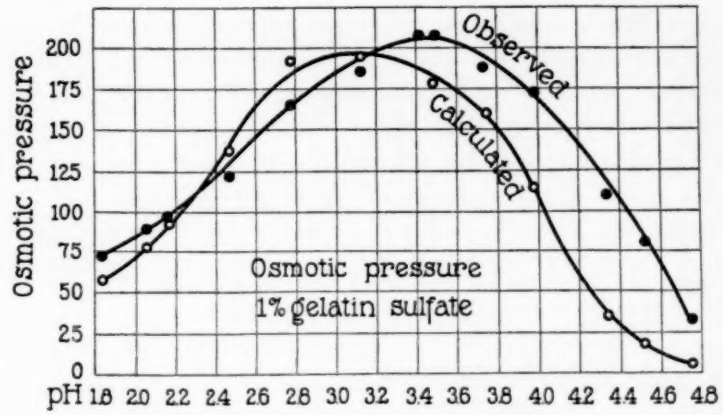


FIG. 4. Comparison of curves for observed and calculated values of osmotic pressure of solutions of 1 per cent gelatin sulfate.

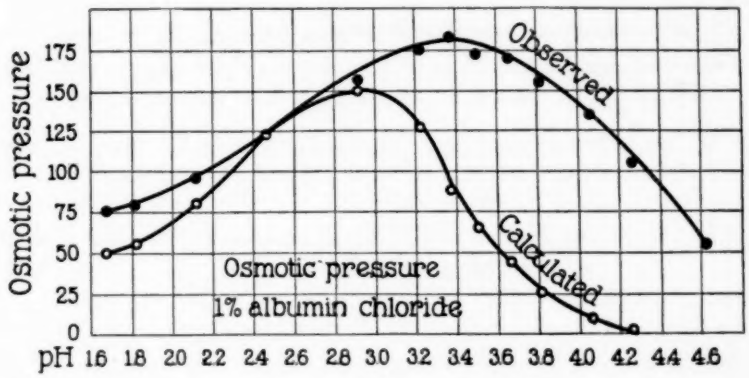


FIG. 5. Comparison of curves for observed and calculated values of osmotic pressure of solutions of 1 per cent crystalline egg albumin chloride.

observed and calculated osmotic pressures of 1 per cent albumin chloride and Fig. 6 the curves for observed and calculated osmotic pressures of 1 per cent albumin sulfate. In Fig. 6 appears the constant difference between the curves for observed and calculated

values which the neglect of the protein value in the calculated curves postulates.

Procter assumes in his theory of swelling that the protein particles do not participate in the osmotic pressure inside the gel, and the writer is willing to admit that the same assumption may be necessary for the osmotic pressure of the protein solutions. The fact that the maximal observed osmotic pressure of gelatin solutions agrees with the maximal pressure calculated, without regard to the possible osmotic pressure caused by the protein ions, seems to agree with Procter's view. In this case, we should have to say that the curves for the observed values coincide with the curves for the calculated values if the latter curves are moved to the right, parallel with themselves.

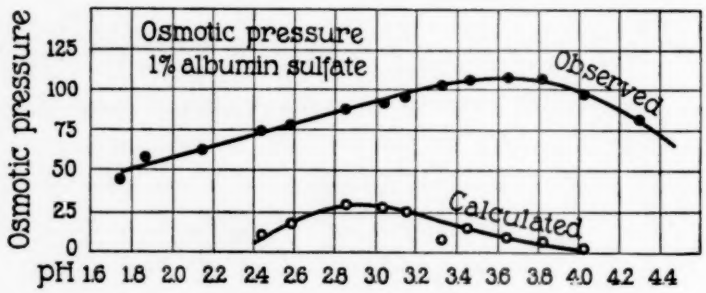


FIG. 6. Comparison of curves for observed and calculated values of osmotic pressure of solutions of 1 per cent crystalline egg albumin sulfate.

### *VI. The Depressing Influence of Neutral Salts on the Osmotic Pressure of a Gelatin Chloride Solution.*

The question arises whether the membrane equilibrium could also be responsible for the depressing influence of salts on the osmotic pressure of protein solutions of a given pH and concentration of originally isoelectric protein. Although Donnan has shown that such a result is to be expected from his theory, the theory does not include all the facts in the case of gelatin chloride solutions. If on one side of the membrane there be a solution of NaCl, on the other side a colloidal salt NaR, where R is the colloidal ion, the real osmotic pressure ($P_o$) of the colloidal solution NaR can, according to Donnan,

be calculated from the observed osmotic pressure ($P_1$) by the following equation,

$$\frac{P_1}{P_o} = \frac{C_1 + C_2}{C_1 + 2C_2}$$

assuming that gelatin is monovalent, which is improbable, where $C_1$ is the concentration of NaCl and $C_2$ that of NaR. If $C_1$ is small compared with $C_2$, $P_1 = \frac{1}{2} P_o$; if, however, $C_2$ is small in comparison with $C_1$, $P_1 = P_o$. It follows from this that the greater the concentration of NaCl (or of any neutral salt) added to a colloidal solution, the smaller the observed osmotic pressure of the colloidal solution becomes, and this is what actually happens. It follows also from this theory that the observed osmotic pressure cannot be depressed below a certain minimum. This seems to agree with the observation that when the solution of the salt reaches the value M/8 a further increase in the concentration of the salt can no longer increase the depressing effect of the salt (see Figs. 1 and 2 in the preceding paper of this series).[2] To this extent Donnan's theory accounts for the depressing action of the salt on the osmotic pressure. A discrepancy between theory and observed value of osmotic pressure arises, however, in the fact that according to the theory the maximal depression caused by a salt should be $\frac{1}{2}$ the real osmotic pressure of the colloidal solution. The observed osmotic pressure of a 1 per cent solution of gelatin chloride of pH 3.5 is about 425 mm. $H_2O$ while the osmotic pressure in the presence of M/8 NaCl is only about 30 mm. According to the theory the latter value should be at least $\frac{1}{2}$ of 425; *i.e.*, 212.

Donnan's theory can only give approximate results in this case since in his theory complicating factors were intentionally ignored for the sake of simplification. Thus the depressing effect of the addition of a neutral salt on the electrolytic dissociation of the electrolyte is not taken into consideration.

Northrop[7] has shown by conductivity measurements that the degree of ionization of gelatin chloride is noticeably depressed when the pH is 2.0 or below and is considerable when the pH is 1.0. There may be other complicating factors besides this depression of ionization.

[7] Northrop, J. H., *J. Gen. Physiol.*, 1920–21, iii, 211.

### *VII. Theoretical Remarks.*

It is of interest to compare the colloidal speculations with the almost quantitative results at which we arrived. All colloidal theories would agree in ascribing the effect of the hydrogen ion concentration or of the valency of the ions with which the protein is in combination to a modification in the state of the protein particles, such as hydratation or degree of dispersion. The hydratation theory

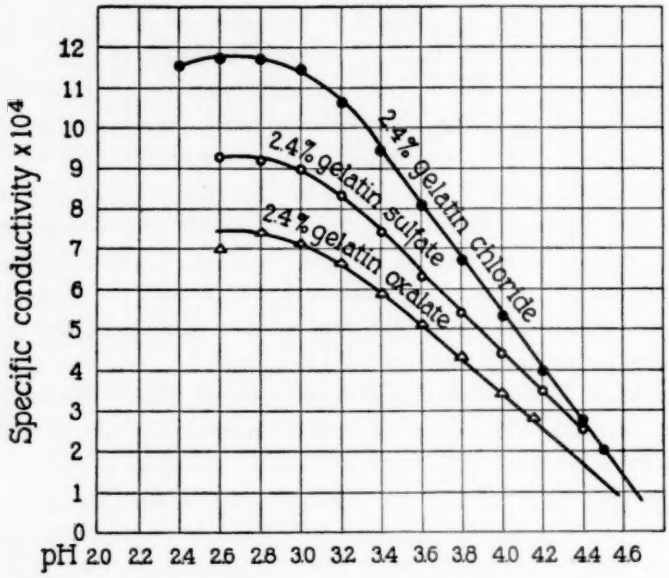


FIG. 7. Curves for the specific conductivity of 2.4 per cent solutions of gelatin chloride, sulfate, and oxalate, showing the entirely different character of these curves from that of the osmotic pressure curves in Figs. 1 and 5 in the preceding paper.

is advocated by Pauli[8] who assumes that the ionized protein particle is strongly hydrated while the non-ionized protein particle is not hydrated. If this were the correct explanation of the peculiar type of curves, such as those reported in Fig. 1 of this paper, we should expect the curves for the specific conductivity of the protein in solution to show a close similarity to the curves in Fig. 1. But such is

[8] Pauli, W., Kolloidchemie der Eiweisskörper, Dresden and Leipsic, 1920.

not the case. In Fig. 7 are given the curves for the specific conductivity of 2.4 per cent solutions of gelatin chloride, sulfate, and oxalate after deduction of the specific conductivity of the free acid in the gelatin solution, as described in a previous paper. A comparison between Fig. 7 and Fig. 1 fails to show any close similarity. In the conductivity curves there is no maximum followed by a drop at pH 3.5, as there is in the osmotic pressure curves. Fig. 8 shows that the

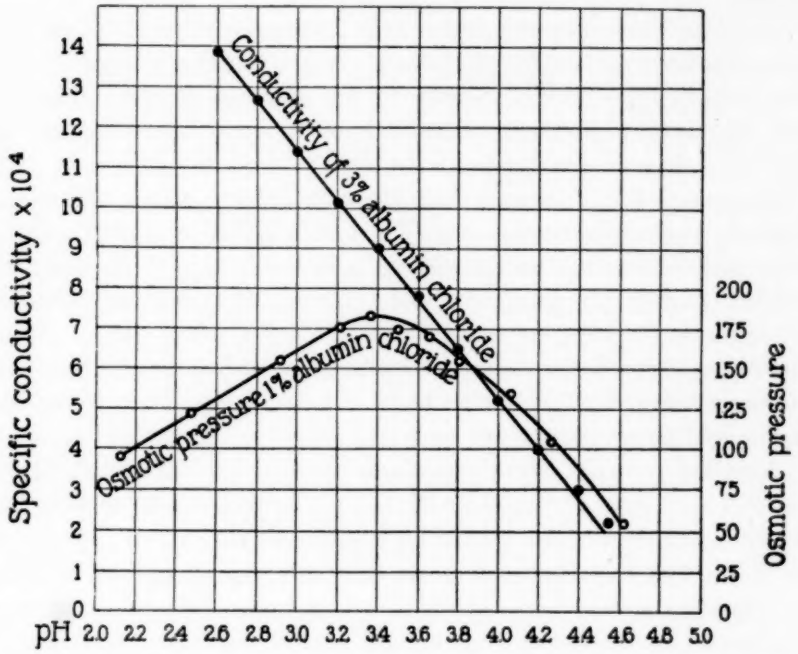
Specific conductivity x 10⁴
Osmotic pressure
Conductivity of 3% albumin chloride
Osmotic pressure 1% albumin chloride
pH
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
0 25 50 75 100 125 150 175 200
2.0 2.2 2.4 2.6 2.8 3.0 3.2 3.4 3.6 3.8 4.0 4.2 4.4 4.6 4.8 5.0

FIG. 8. Comparison of conductivity curve and osmotic pressure curve for albumin chloride, showing the entirely different character of the two curves.

difference between the conductivity curve and the osmotic pressure curve is still more pronounced in the case of albumin chloride.

Manabe and Matula,[9] who claim to have proved Pauli's theory, speak of a maximum of the physical properties of protein solutions at pH 2.0 or 2.1. There seems to be some misunderstanding, since the maximum for osmotic pressure of solutions of protein-acid salts;

[9] Manabe, K., and Matula, J., *Biochem. Z.*, 1913, lii, 369.

*e.g.*, gelatin or crystalline egg albumin, lies at a pH of 3.5 or 3.4, and for casein at a pH above 3.0. The maximal swelling lies at a pH of gelatin of about 3.2 or 3.3. Those who state that it lies at a lower pH (*e.g.* Michaelis[10]) must have mistaken the pH of the supernatant liquid for the pH of the solid gelatin, thus ignoring the effects of the Donnan equilibrium. The maximum for the viscosity of gelatin-acid salts lies at pH of 3.0 and for the viscosity of casein chloride or phosphate at pH 3.0 or above.

Northrop[7] has observed that a drop in the conductivity of gelatin solutions occurs when the pH falls below 2.0, but this cannot explain the drop in the osmotic pressure curves observed at pH above 3.0, and Northrop's results agree entirely with my own in not having noticed a drop in the conductivity curves at pH 3.0 or 3.5.

Moreover, Fig. 7 shows that there is only a slight difference between the conductivity curves for gelatin sulfate and gelatin chloride, while there is a greater difference between the conductivity of gelatin chloride and gelatin oxalate. All this disagrees entirely with the osmotic pressure curves in Fig. 5 in the preceding paper. Furthermore, the idea of a noticeable hydratation of the protein ion seems to be no longer tenable on the basis of Lorenz[11] and Born's[12] experiments and conclusions.

A second colloidal hypothesis would lead us to assume that variations in the degree of dispersion of the protein particles are responsible for the osmotic pressure curves represented in Fig. 1. We need not dwell on this hypothesis since we have no way of putting it to a quantitative test.

The results of this paper show that if we assume the correctness of Donnan's theory of membrane equilibrium the characteristic influences of pH and valency on the osmotic pressure appear as a necessary consequence of the theory; with the exception of the two minor differences discussed in Chapter IV. Donnan's theory leads to a view radically different from all colloidal speculations since on the basis of this theory the variations in osmotic pressure depend on the

[10] Michaelis, L., Praktikum der physikalischen Chemie insbesondere der Kolloidchemie, Berlin, 1921.

[11] Lorenz, R., *Z. Elektrochem.*, 1920, xxvi, 424.

[12] Born, M., *Z. Elektrochem.*, 1920, xxvi, 401.

unequal distribution of the crystalloidal ions on the opposite sides of the membrane and not on variations in so called colloidal properties of proteins. The quantity which changes with the pH and the valency of the anion of a protein-acid salt is on this assumption not the degree of hydratation or dispersion of the protein particles but the value pH inside minus pH outside, as stated more fully in the two preceding papers.[1]

SUMMARY.

1. It had been shown in previous publications that the osmotic pressure of a 1 per cent solution of a protein-acid salt varies in a characteristic way with the hydrogen ion concentration of the solution, the osmotic pressure having a minimum at the isoelectric point, rising steeply with a decrease in pH until a maximum is reached at pH of 3.4 or 3.5 (in the case of gelatin and crystalline egg albumin), this maximum being followed by a steep drop in the osmotic pressure with a further decrease in the pH of the gelatin or albumin solution. In this paper it is shown that (aside from two minor discrepancies) we can calculate this effect of the pH on the osmotic pressure of a protein-acid salt by assuming that the pH effect is due to that unequal distribution of crystalloidal ions (in particular free acid) on both sides of the membrane which Donnan's theory of membrane equilibrium demands.

2. It had been shown in preceding papers that only the valency but not the nature of the ion (aside from its valency) with which a protein is in combination has any effect upon the osmotic pressure of the solution of the protein; and that the osmotic pressure of a gelatin-acid salt with a monovalent anion (*e.g.* Cl, $NO_3$, acetate, $H_2PO_4$, $HC_2O_4$, etc.) is about twice or perhaps a trifle more than twice as high as the osmotic pressure of gelatin sulfate where the anion is bivalent; assuming that the pH and gelatin concentrations of all the solutions are the same.

It is shown in this paper that we can calculate with a fair degree of accuracy this valency effect on the assumption that it is due to the influence of the valency of the anion of a gelatin-acid salt on that relative distribution of the free acid on both sides of the membrane which Donnan's theory of membrane equilibrium demands.

3. The curves of the observed values of the osmotic pressure show two constant minor deviations from the curves of the calculated osmotic pressure. One of these deviations consists in the fact that the values of the ascending branch of the calculated curves are lower than the corresponding values in the curves for the observed osmotic pressure, and the other deviation consists in the fact that the drop in the curves of calculated values occurs at a lower pH than the drop in the curves of the observed values.